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RESEARCH PAPER

High level production of itaconic acid at low pH by *Ustilago maydis* with fed-batch fermentation

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Abstract

The metabolically engineered plant pathogen *Ustilago maydis* MB215 Δ*cyp3* P_{etef}*ria1* has been cultivated to produce more than 80 g/L itaconate in 16 L scale pH and temperature controlled fermentation, in fed-batch mode with two successive feedings. The effect of pH as well as successive rounds of feeding has been quantified via elemental balances. Volumetric itaconic acid productivity gradually decreased with successive glucose feedings with increasing itaconic titers, with nearly constant product yield. Extracellular pH was decreased from 6 down to 3.5 and the fermentation was characterized in specific uptake, production, and growth rates. Notable is that the biomass composition changes significantly from growth phase to itaconic acid production phase, carbon content increases from 42% to around 62%. Despite the gradual decrease in itaconic acid levels with decreasing pH (nearly 50% decrease in itaconic acid at pH 3.5, compared to pH 6), significant itaconate production is still observed at pH 4 (around 63 g/L). Biomass yield remained nearly constant until pH 4. Taken together, these results strongly illustrate the potential of engineered *Ustilago maydis* in itaconate production at commercial levels.

Keywords Itaconic acid · *Ustilago maydis* · Fed-batch fermentation · Weak acid stress · Low pH cultivation

Introduction

Organic (weak) acids are ubiquitous in various area, ranging from biobased materials, biofuels, and medical to food applications. So far, various organic acids have been and are still being produced, e.g. citric, propionic, succinic, and adipic in both academic, and industrial studies [1–3].

To produce the weak organic acids, a variety of industrial workhorses both eukaryotic and prokaryotic are being used. *Saccharomyces cerevisiae,* enjoying the available know-how on its physiology, genome, fermentation characteristics, and recombinant DNA technology has been used to produce succinic acid [4–7]. Various other bacterial or fungal hosts (e.g. *Mannheimia succinoproducens, Aspergillus niger*) are also used for production of organic acids [8–12]. While the available genetic toolbox renders known industrial hosts (*S. cerevisiae, E. coli*) favorable for tailor-made production, new hosts are still highly valuable, not only to enable economically attractive production, but also to allow the discovery of potentially novel production and/or secretion pathways, thereby improving our understanding on such systems [13, 14].

Itaconic acid (IA, Methylenesuccinic acid) is a commercially important unsaturated dicarbonic organic acid, with interesting features and applications in a range of industries, in particular bio-based materials [15, 16]. It can easily be incorporated into polymers and may serve as a substitute for petrochemical-based acrylic or methacrylic acid with applications in polymer industry, papermaking, and waste water treatment [17].

Conventionally, *Aspergillus terreus* is used to produce itaconic acid. However, the filamentous morphology of *Aspergillus* species presents challenges in particular during production caused by decreased oxygen transfer, sensitivity to hydromechanical stress, and sensitivity to mg-range

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medium impurities [18, 19]. Alternative wild-type production hosts have been identified, e.g., *Pseudozyma*, *Candida* and *Ustilago* strains [20–22].

Ustilago strains have been shown to be a good candidates for itaconic acid production. They grow unicellularly in their haploid form (yeast-like growth), eliminating the disadvantages of filamentous fungi and making them useful for production using lignocellulosic carbon sources [17, 23]. *Ustilago maydis* is a Basidiomycete fungal pathogen of maize and teosinte and has been recently used to produce a variety of organic acids, glycolipids, and polyols, with several favorable characteristics such as high-stress resistance and insensitivity to medium impurities [19, 23–27]. Its genome (~ 20 Mb) has been recently published ([26], NCBI accession nr: GCF_000328475.2) and is larger than that of *S. cerevisiae*.

Itaconic organic acid production by *Ustilago* is affected by both the strain and culture conditions. Cultivation conditions such as temperature, pH, growth-limiting nutrients, and aeration significantly affect the product range and productivity of the organic acids. Generally, a nitrogen limitation is used to efficiently induce itaconic acid production in Ustilaginaceae [19]. Due to the activity of the promoters of itaconate cluster which are induced by nitrogen depletion, itaconate is produced along with malate and succinate after nitrogen depletion [28, 29]. Secondary growth continues also after nitrogen depletion, characterized by intracellular lipid formation leading to swollen cells and the use of intracellular nitrogen pools [17]. Under nitrogen limitation, cell composition changes and hydrocarbons accumulate, leading to higher C/N ratio with larger cell sizes [17, 19, 30]. Osmolarity causes delayed exponential growth phase and decreased maximum oxygen transfer rate, so decreased growth in high osmolarity might accelerate IA secretion [19]. Finally, pH stress causes metabolic shift from acid production to polyol and glycolipid production, so careful pH control and the selection nature of acid used as buffer are essential [19, 31].

Several recent works describe the selection and engineering of itaconic acid hyper-producing *Ustilago* strains. *U. maydis* MB215 (= DSM 17144) was selected as best production strain out of 68 Ustilaginaceae [17]. The itaconate production pathway and associated genes were characterized in this strain [32]. This knowledge was applied to enhance yield, titer, and rate through the overexpression of the transcriptional regulator *ria1* and the deletion of the itaconate oxidase *cyp3* [32, 33], enabling the production of up to 63 g/L itaconate at benchtop scale. Further optimization targets include the MttA mitochondrial transporter from *A. terreus* [34], and the control of morphology and pathogenicity through the deletion of the regulator gene *fuz7*, especially in the alternative host *Ustilago cynodontis*, which is more pH-tolerant than *U. maydis* [35].

An efficient, robust producer strain (high specific production rate, resilient to stress, with minimal by-products) is undoubtedly central for an economically viable fermentative production [36]. Yet, once the promising host has been obtained and tested in bench-top scale, a fermentation, and possibly downstream strategy, suitable for that host's needs, must be adopted [37–39]. Considering this, the aim of this work was to design and implement a fermentation strategy to produce around 80 g/L itaconic acid using previously obtained metabolically engineered *U. maydis* MB215 Δ*cyp3* P_{etef}*ria1* [32] cells using glucose as the carbon source. The fermentation characteristics of the cells have been quantified by yield, final titer, and the so-called qRates, i.e. specific substrate consumption, itaconate production, growth rates as well as specific oxygen uptake (OUR) and carbon dioxide production (CPR) rates. Related to that, the elemental balances for carbon, nitrogen as well as degree of reduction are used to assess the quality of the fermentation process.

Materials and methods

Strains

Wild-type *U. maydis* MB215 (DSM 17144) used in this study was obtained from DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures) culture collection; recombinant strain *U. maydis* MB215 Δ*cyp3* P_{etef} *ria1* was constructed by Geiser et al. [32, 40]. The strains were stored at – 80 ℃ in 80% (v/v) glycerol stocks. Stock cultures were grown on YEPS medium consisting of 10 g yeast extract, 20 g peptone, and 20 g sucrose per liter and 20 g agar for solid media.

Growth, production, and feeding media

Pre-cultivation

Cells, from glycerol stocks, were activated on YEPS agar plates for 24 h at 30 ℃. For the preparation of the bioreactor inoculum, a two-stage liquid precultivation procedure was used. In the first stage, cells were inoculated to YEPS liquid medium incubated in shake-flasks at 30 ℃ for 24 h. Afterwards, Modified Tabuchi Medium (MTM) [23], consisting of 30 g/L glucose, 1.6 g/L NH_4Cl, 0.5 g/L KH_2PO_4, 0.41 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L yeast extract, 0.14 g/L $FeSO_4 \cdot 7H_2O$ was inoculated with 50 mL YEPS grown cells for a final volume of 300 mL in each shake-flask. After 24 h at 30 ^{0}C, precultures were inoculated to the bioreactor to achieve an initial OD_{600} of 1.0.

Cultivation at 16 L Bioreactor

Bioreactor scale fermentations were performed using a production medium containing approximately 190 g/L glucose, 4 g/L NH_4Cl, 1 g/L yeast extract, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.01 $FeSO_4{\cdot}7H_2O$, 0.5 g/L KH_2PO_4, 1 mL/L vitamin solution, and 1 mL/L trace element solution [32]. The vitamin solution containing 0.05 g/L D-biotin, 1 g/L D-calcium panthotenate, 1 g/L nicotinic acid, 25 g/L myo-inositol, 1 g/L thiamine hydrochloride, 1 g/L pyridoxol hydrochloride, 0.2 g/L para-aminobenzoic acid, and trace element solution containing 1.5 g/L EDTA, 0.45 g/L $ZnSO_4{\cdot}7H_2O$, 0.10 g/L $MnCl_2{\cdot}4H_2O$, 0.03 g/L $CoCl_2{\cdot}6H_2O$, 0.03 g/L $CuSO_4{\cdot}5H_2O$, 0.04 g/L $Na_2MoO_4{\cdot}2H_2O$, 0.45 g/L $CaCl_2{\cdot}2H_2O$, 0.3 g/L $FeSO_4{\cdot}7H_2O$, 0.10 g/L H_3BO_3, and 0.01 g/L KI were used as previously described by Geiser et al. [17]. Glucose additions were made by a sterile stock glucose solution with a concentration of 700 g/L. All chemicals were purchased from Merck (Germany), Sigma-Aldrich (USA), VWR BDH Chemicals (USA) and BD Difco (USA).

Fermentation conditions

Fermentations were conducted in 16-L stainless steel stirred tank bioreactor (NLF22, Bioengineering, Switzerland) with a cooler at the air outlet to avoid evaporation and having rushton 6-blade impellers. Initial working volume was used as 8 L where temperature was controlled at 30 ^{0}C. During fermentations, dissolved oxygen levels were maintained above 30% by controlling the stirrer speed between 700–900 rpm while the aeration rate was fixed at 3 L/min where 0.3–0.4 vvm was achieved. pH was adjusted by automatic addition of 10 M NaOH (Merck, Germany) to pH 6 for the development of the feeding strategy and the wild-type productions, whereas for the investigation of the effects of pH, pH 3.5, 4, 5, and 6 were used.

Analytical methods

Quantification of cell dry weight and cell composition

At selected time intervals, fermentation broth was centrifuged at 10,000 rpm for 10 min to separate cells from the fermentation medium. Cell dry weights were measured by drying to constant weight with an electrical moisture analyzer (MA 150Q, Sartorius, Germany) at 105 °C. Cell C, H, N contents were analyzed by Dumas method using LECO Truspec CHN system (LECO, USA); ash contents were determined by a laboratory chamber furnace (CWF 1200, Carbolit Gero, UK) with a method of heating the dried cell sample to 600 ℃ for 16–18 h until constant weight achieved.

Quantification of sugars, organic acids, nitrogen, and phoshate

For the quantification of the sugars, organic acids and nitrogen in the media, supernatant samples were filtered through a 0.45-µm filter (Minisart RC, Sartorius, Germany). Itaconic acid, glucose and other acid concentrations were analyzed by HPLC (1100 Series, Agilent Technologies, USA). Total nitrogen and ammonia concentrations were determined by Kjeldahl and distillation-titration methods, respectively [41]. Phosphate contents were analys***ed by a colorimetric method using *LCK 349 Phosphate Kit* produced by HACH LANGE, Germany. Osmotic pressure was determined by an automatic cryoscopic osmometer (OSMOMAT 030, Gonotec, Germany). Dissolved CO_2 concentrations were estimated via alkalinity measurements as $CaCO_3$ equivalent using acidimetric-titration method in which the samples were titrated with 0.1 N H_2SO_4 to pH:4.0. Due to the presence of high levels of itaconic (and other organic) acid(s), this alkalinity measurement needs to be corrected. Focusing on the itaconic acid, $CaCO_3$ alkalinity is total alkalinity minus the alkalinity due to itaconic acid as follows:

$$CaCO_3 \text{ alk} = \text{Total alk} - \pi_{pH=4} \cdot \frac{MW_{CaCO_3}/\gamma_{CaCO_3}}{MW_{Ita}/\gamma_{Ita}} \cdot [Ita],$$

where $\pi_{pH=4}$ is the fraction of undissociated itaconic acid at pH 4 equal to 0.4, and second term is the ratio of molecular weights of $CaCO_3$ to itaconic acid, corrected for their valence, equal to 0.77. In the above equation, all entities are in g/L.

Results

Comparison of wild-type and mutant strains for itaconic acid production fermentation

U. maydis MB215 WT and the Δ*cyp3* P_{etef}*ria1* strain were cultivated in fed-batch mode, to compare the growth and itaconic acid production performance. Overall, following the Total Kjehldahl Nitrogen (TKN) decrease in the first 48 h, the biomass levels stabilizes around 25–30 g/L, and the glucose fed is used for acid production in both WT and mutant strain. The glucose consumption profile is found to be similar for both strains. As for organic acid production, WT strain produces mixed organic acids, while the mutant strain produce itaconic acid without detectable byproducts (Fig. 1). In HPLC profile of the supernatant, a peak that does not match the available organic acid standards (succinic, malic, itaconic, fumaric, citric acids) is obtained

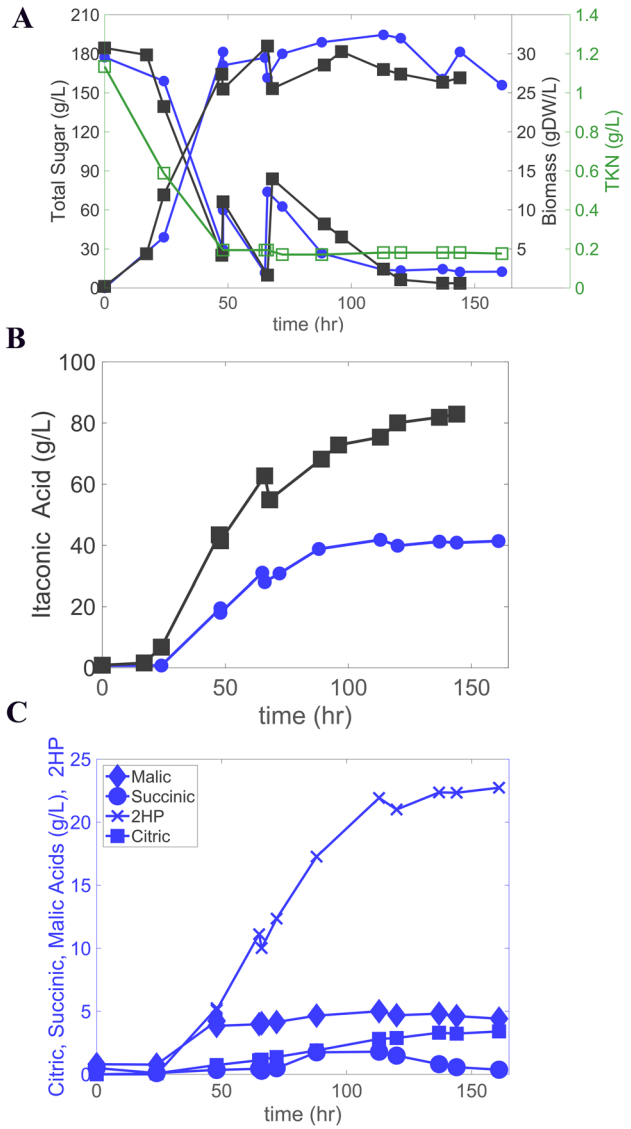

Fig. 1 Comparison of the fermentation and itaconic acid production profile for the wild-type (blue lines in all plots) and Δcyp3 P_{etef}ria1 mutant (black lines in all plots) strains of Ustilago maydis. **a** glucose, biomass (filled circles), and TKN levels (squares), **b** itaconic acid, **c** organic acids produced other than itaconic acid. The mutant has no significant acid production other than itaconic acid. Shown are representative data from single fermentations (color figure online)

and following Geiser et al. [32] the peak is attributed to 2-hydroxyparaconate and values should be taken as rough estimates only.

Fermentation strategy: feeding time, number of feeding cycles

The following experiments were performed with *U. maydis* MB215 Δ*cyp3* P_{etef}*ria1* cells to test itaconic acid production in 16 L bioreactor in fed-batch mode. Based on the results of preliminary experiments in batch mode with various glucose concentrations, no significant substrate inhibition was observed as the initial glucose uptake rate was the same for the substrate range studied (50–200 g/L, data not shown). Following this, pulse feeding was adopted. Figure 2 represents the results of a typical fed-batch itaconic acid fermentation with five feeding cycles.

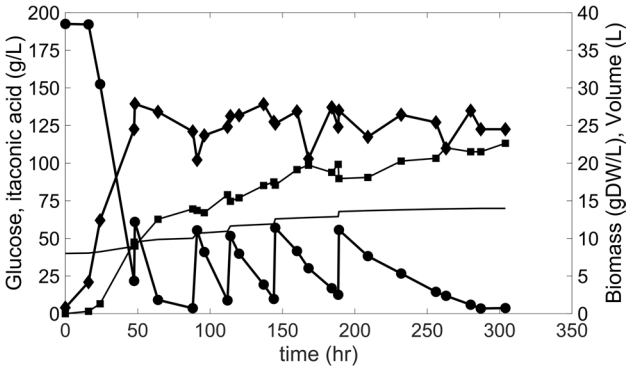

Fig. 2 Fermentation data on fed-batch cultivation (with pulse feeding) of U. maydis MB215 Δcyp3 P_{etef}ria1 at a pH controlled at 6. Glucose (circles), itaconic acid (squares), biomass (diamonds) are presented in g/L and culture volume in L (dashed line)

Glucose was pulse-fed into the fermenter when, residual glucose concentration was around 5–10 g/L as consuming the remaining glucose would result in extended fermentation time and repeated feeding also dilutes the product, being collectively undesired features. The specific glucose consumption rate changes minimally in the first two cycles, while in the third cycle, all three specific q-rates (growth, glucose consumption and itaconate production) decrease significantly while the yield was nearly constant. Based on this data, the base case scenario is set to two feeding cycles, corresponding to approximately 48th and 96th hours for the first and second pulse feeds.

For the base case scenario, *U. maydis* MB215 Δ*cyp3* P_{etef}*ria1* cultivated at pH 6, with two pulse feeding cycles, the fermentation profile including the offline and online data is given in Fig. 3. Overall, by using the described fermentation strategy, a commercially relevant itaconic acid level (80–90 g/L) is reached within 150 h with two feeding cycles. Furthermore, the strain produces no other significant organic acids. Biomass is produced in the first 50 h (in line with TKN measurements), later sustaining constant level around 21–22 g/L. As for the offgas data, the CO_2 level gradually increases due to intensive growth at the beginning followed by a decrease during itaconate production at the second stage after 48 h (Fig. 3). Overall, the substrate is only used for production of biomass, itaconate, and cellular maintenance, with the carbon balance being closed with a gap of less than 10%.

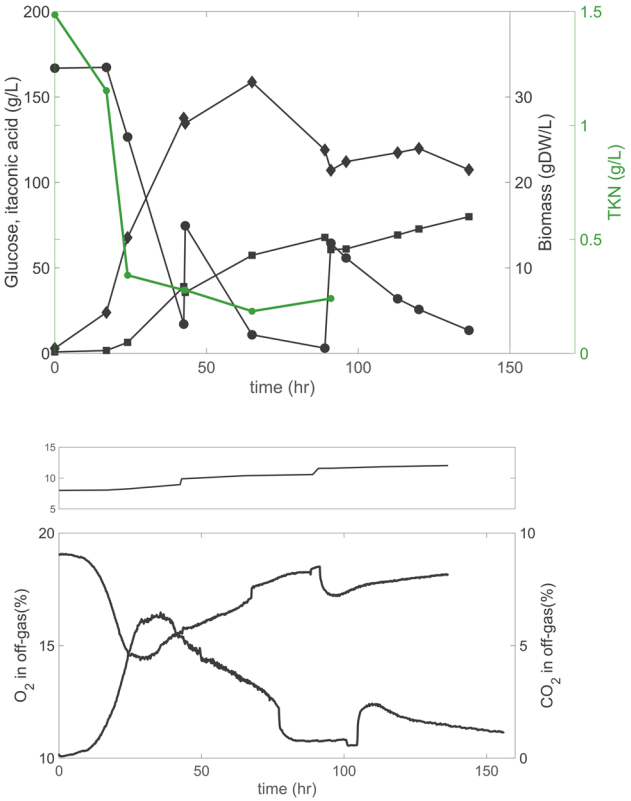

Fig. 3 Fermentation data on fed-batch cultivation of U. maydis MB215 Δcyp3 P_{etef}ria1 for the base case scenario (pH 6, feeding with two successive substrate pulses) as **a** glucose (circles), itaconic acid (squares), biomass (diamonds) in g/L, and TKN (filled circles) in g/L and **b** off-gas data as CO_2 and O_2 fractions of the offgas, the upper panel plots the working volume of fermentor (L), indicating each feeding time

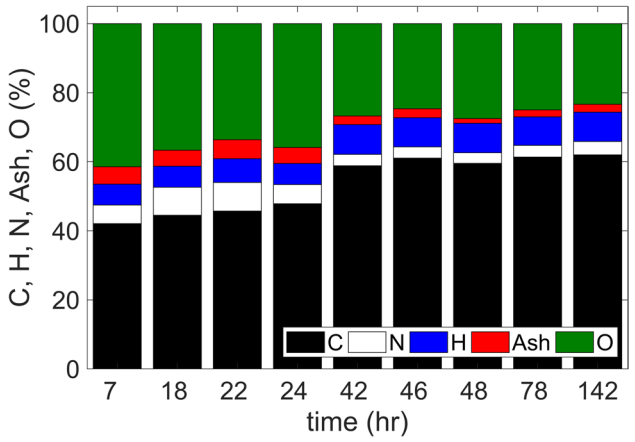

Fig. 4 Changes in the elemental composition of U. maydis MB215 Δcyp3 P_{etef}ria1 cells during fermentation. The carbon, nitrogen, hydrogen, and ash are measured while oxygen fraction is calculated to have the sum equal to 100

Changes in the elemental composition of biomass:

Itaconic acid fermentation with *U. maydis* consists of two distinct stages: (1) biomass growth and itaconic production and (2) itaconic acid production alone. Throughout the fermentation, the morphology of the cells changes. At the initial stage of the fermentation, the cells are yeast-like, while at a later stage, they elongate and transform into longer filaments. Additionally, the TKN measurements over time yielded on average a lower nitrogen content than what can be expected from an average biomass composition ($CH_{1.65}O_{0.54}N_{0.14}$ [42]). This is apparent as a gap between TKN and biomass measurements, which further resulted in a gap in carbon and degrees of reduction balances. To understand this, we quantified C, N, and H content of *U. maydis* biomass samples taken from different stages of the fermentation (Fig. 4). When the cells cease secondary growth, exhibited by relatively constant CDW measurements (IA production only, later than 48th hour) the carbon content of the cells increased by 47% (from 0.42 to 0.62), while the nitrogen content decreased by 28% (from 0.053 to 0.038). Such a decrease in nitrogen content of biomass has been previously pointed by von Stockar and Liu, where algea, typically storing oils, contain considerably less nitrogen than average yeast [42, 43]. In our current work, the reducing power of biomass, calculated from the determined elemental composition, increases. The increase is due to increased carbon percentage and this increase points to lipid accumulation, in line with previous studies with *Ustilago maydis* under nitrogen starvation conditions [44–46].

Effect of pH on itaconic acid production under successive pulse-feeding

To observe the impact of cultivation at low pH on growth and itaconic acid production, cells were cultivated at pH 6, 5, and 4 and the fermentation is monitored both in its growth and itaconic production as well as corresponding rates (Fig. 5). The carbon- and degree of reduction balances are also reported (Fig. 6a). Overall, the recoveries are within accepted ranges, i.e. within 10–15% margin. Remaining carbon and degree of reduction gap is attributed to visually observed glycolipids secreted to fermentation medium, also reported in the literature [44, 45].

The general trend is that, as pH decreases, *U. maydis* cells grow slower and produce less itaconic acid (Fig. 5), in line with several previous reports with different hosts. In the experiments reported here, the biomass level after 48 h decreased around 3.5 gDW/L for pH from 6 to 5, while the biomass dropped more than 10 gDW/L for pH from 6 to 4, the change being statistically significant considering the batch-to-batch difference as well ($p = 5.4\ 10^{-4}$).

Overall, as the pH decreases, a decrease in yields of biomass and itaconic acid is observed (Fig. 6a), until pH

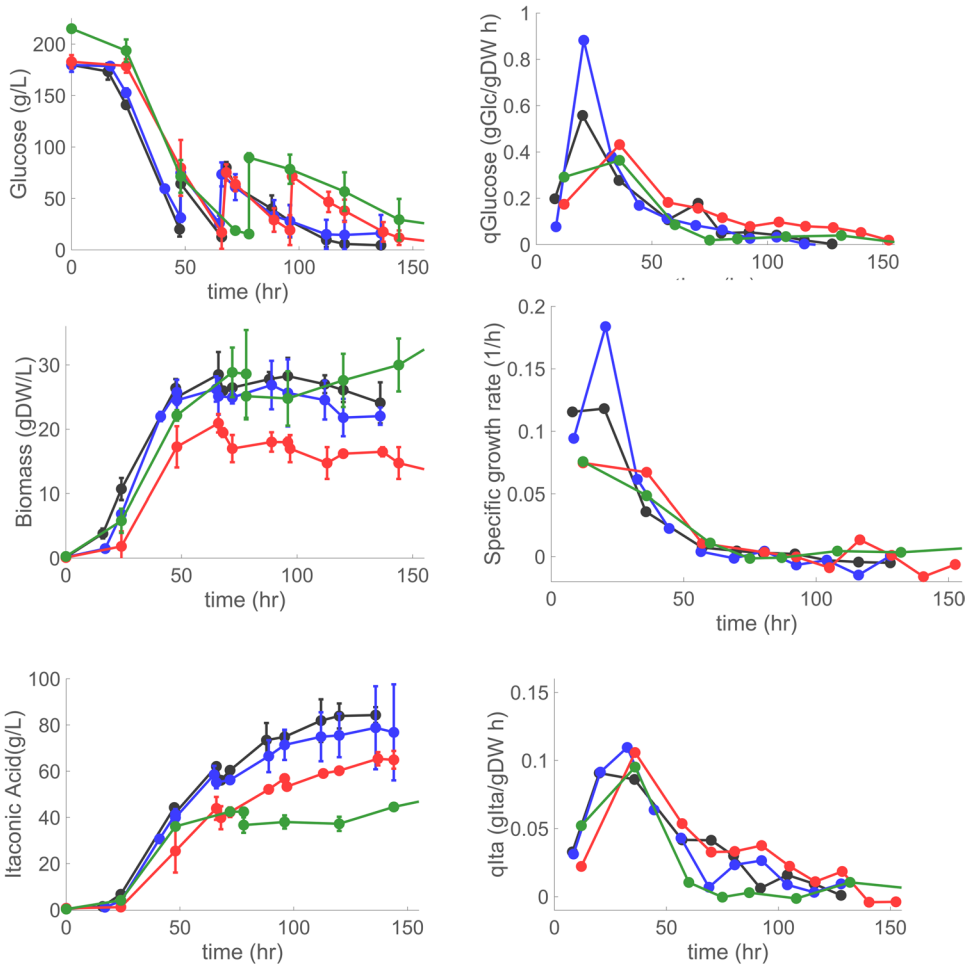

Fig. 5 The effect of extracellular pH on the growth and itaconic acid production of U. maydis MB215 Δcyp3 P_{etef}ria1. Left panel describes the measured concentrations of glucose, biomass, and itaconic acid while the right panel depicts the calculated biomass specific substrate consumption, growth, and itaconic acid production rates. In both panels, the extracellular pH was kept at 6 (black),5 (blue),4 (red), and 3.5 (green). Despite higher specific glucose uptake rate, the cells grow and produce itaconic acid at an approximately same rate, pointing the fact that the excess glucose would be used for maintenance due to weak acid stress. Fermentation were performed in at least duplicates, error bars depict the standard deviation from the mean (color figure online)

4. The cells, upon acid stress, consume more oxygen, and produce more CO2 (higher specific OUR and CPR), which is expected since the cells would need additional energy for maintenance of their pH homeostasis (Fig. 6) [13]. Interestingly, at pH 3.5, growth rate and biomass yield are comparable if not higher than those at pH 6; however the itaconic acid production rates as well as itaconic yields are considerably lower.

Effect of nutrient limitation on itaconic acid production

Generally, organic acids are produced under nitrogen and/or phosphate limitation to control the growth and thereby redirect carbon flow to the desired product. In this respect, itaconic acid production by *A. terreus* is reported to work optimally under phosphate-limited conditions at sugar concentrations between 100 and 150 g/L [16] though Krull et al. reported similar itaconic acid yields under various phosphate conditions [47]. In contrast, nitrogen limitation is reported to induce itaconate production in *U. maydis* [28]. Indeed, at the initial stage of the fermentations with *U. maydis* MB215 Δ*cyp3* P_{etef}*ria1* (first 24 h), the cells mainly grow while nitrogen levels decrease, followed by the production phase where mainly itaconic acid is produced (Figs. 1 and 3). Soon after the second glucose feeding, we detected significant amounts of nitrogen and phosphate in the medium, possibly due to protein secretion, which is known to occur in *U. maydis* cultures with similar conditions [48] (Fig. 7). From thereon, despite no apparent nutrient limitation, the cells continue itaconic acid production in the presence of phosphate and nitrogen, while keeping the biomass level constant. Last, the dissolved CO_2 level, measured via the net $CaCO_3$ alkalinity illustrates that, near neutral pH there is enough dissolved CO_2 in the medium that the cell may use, e.g. for increased PEP carboxykinase or pyruvate carboxylase activity, two enzymes are typically targeted to increased organic acid production [49, 50].

Discussion

In this work, we reported the production of over 80 g/L itaconic acid, using *U. maydis* MB215 Δ*cyp3* P_{etef}*ria1* in pulse fed-batch mode with two successive feedings, and

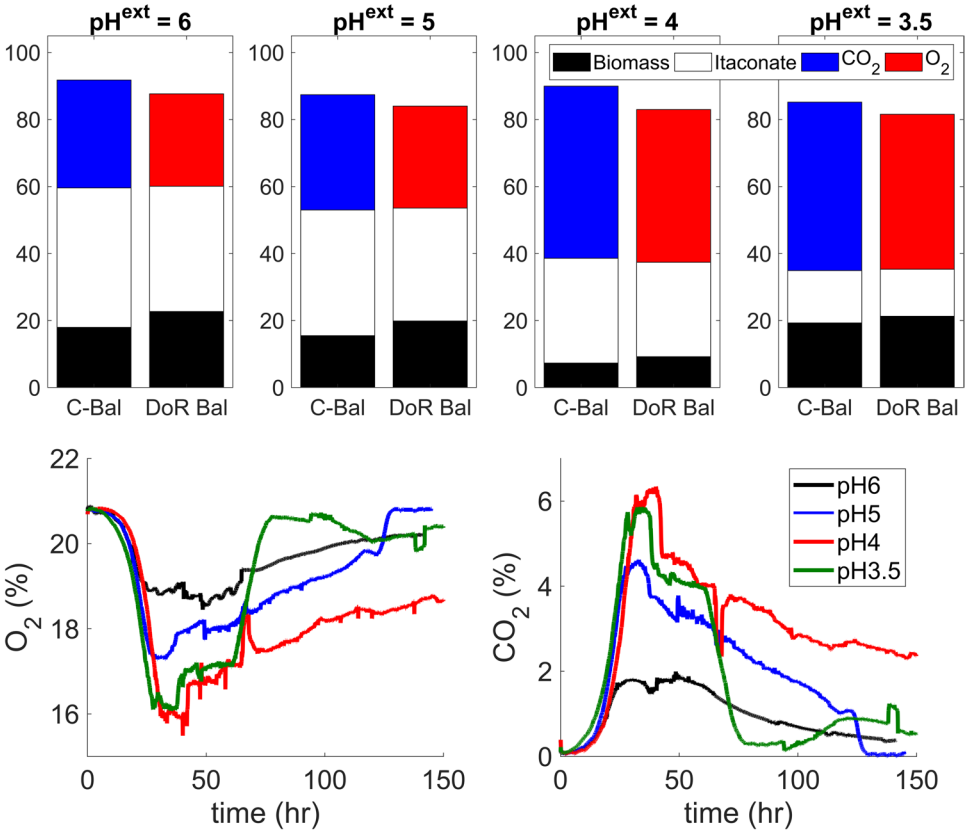

Fig. 6 Probing the response to weak acid stress in U. maydis. **a** Carbon and Degrees of Reduction recoveries for the corresponding fermentations at different extracellular pH. The relative contributions of biomass (black), itaconic acid (white), carbon dioxide (blue), and oxygen (red) for each balance is given. **b** offgas data from U. maydis MB215 Δcyp3 P_{etef}ria1 fermentations at different extracellular pH conditions. Colors correspond to Fig. 5 (color figure online)

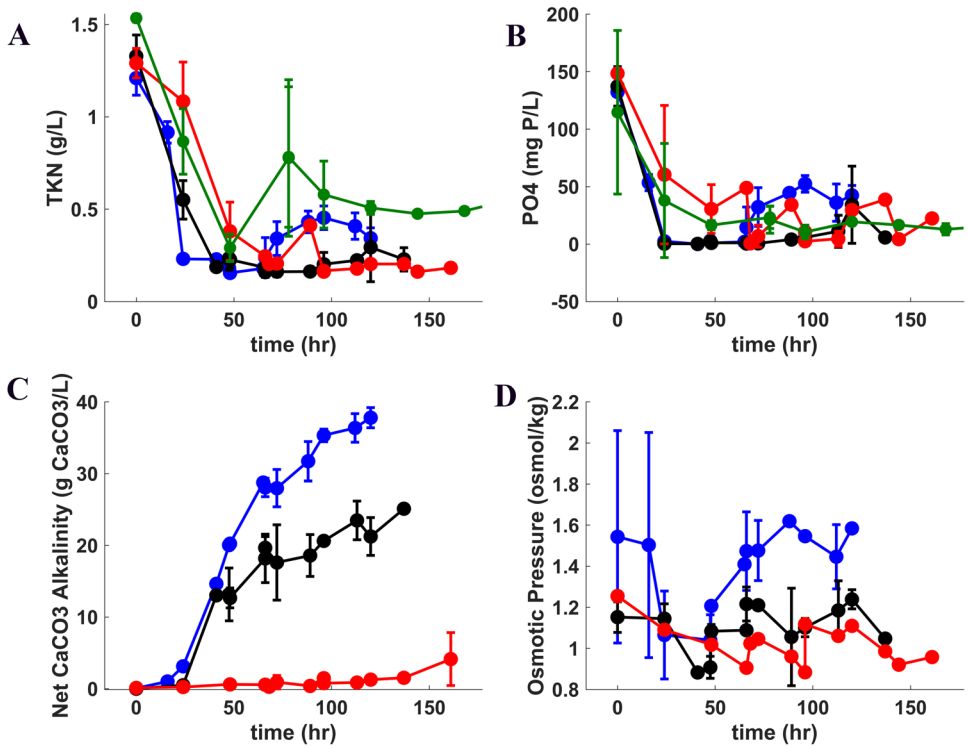

Fig. 7 **a–b** Dynamics of Nitrogen and Phosphate levels for different extracellular pH levels. **c–d** Alkalinity and Osmotic pressure as a function of extracellular pH. In both panels, the extracellular pH is kept at 6 (black), 5 (blue), 4 (red), and 3.5 (green). For CaCO3 alkalinity, the experiments at pH 3.5 are omitted as the titration in alkalinity test is performed down to pH 4, beyond which no bicarbonate is present in the medium. Fermentation were performed in at least duplicates, error bars depict the standard deviation from the mean (color figure online)

Table 1 Comparison of itaconic acid fermentation performances

Ref	Host	C_{IAmax} (g/L)	Productivity IA (g/L/h)	P_{IAmax} (g/L/h)	$Y_{IA/S}$ (g/g)	Time (d)
Kuenz et al. [52]	*A. terreus DSM-23081*	86.2	0.51	1.2	0.62	7
Hevekerl et al. [51]	*A. terreus DSM-23081*	87–146	0.41–1.15	1.2–2.64	0.53–0.59	4.7–12.6
Krull et al. [47]	*A. terreus DSM-23081*	150–160	0.64–0.99	1.7–1.9	0.46–0.56	6.8–9.7
Geiser et al. [32]	*U. maydis* Δ *cyp3_*P_{etef}*-ria1*	63.2	0.38	–	0.23	6.8
This work, high IA ferm	*U. maydis* Δ *cyp3_*P_{etef}*-ria1*	113	0.37	1.00	0.38	12.7
This work, pH:6	*U. maydis* Δ *cyp3_*P_{etef}*-ria1*	88.4	0.79	0.95	0.41	4.7
This work, pH:5	*U. maydis* Δ *cyp3_*P_{etef}*-ria1*	73	0.65	0.95	0.31	4.7
This work, pH:4	*U. maydis* Δ *cyp3_*P_{etef}*-ria1*	68.4	0.42	0.72	0.27	6.7
This work, pH:3.5	*U. maydis* Δ *cyp3_*P_{etef}*-ria1*	48	0.28	0.75	0.20	7

over 110 g/L with five successive feedings. We compare the obtained fermentation performance with earlier works, whereby *A. terreus* is used for itaconic acid production at (near-)commercial levels [47, 51, 52] (Table 1). Taken together, significant levels of itaconic acid are produced using this fermentation process in a relatively short fermentation time with high volumetric productivity. Surprisingly, compared to previously published *U. maydis MB215 Δcyp3* P_{etef}*ria1* fermentations at benchtop scale [32], the yield and productivity in the current work is higher, albeit being similar in scale (around tenfold increase 1.3 L in [32] vs. 16 L, this work). The exact reason for this large difference is currently unknown, but may be related either to small differences in the pre-cultivation such as the omission of MES buffer and lower glucose concentration, or to scale-up effects which may lead to local heterogeneities in oxygen supply. Especially the latter may have a significant effect on itaconic acid production, as was previously shown fermentations of the closely related *Pseudozyma* under a regime of oscillating dissolved oxygen concentration [53].

In analyzing the fermentation data with successive glucose feeds, the glucose consumption as well as itaconate production rates of the *U. maydis* MB215 Δ*cyp3* P_{etef}*ria1* cells in each feeding cycle is important parameters, since they allow to decide whether successive pulse feeding is possible, with the same biomass. We noted that after second glucose feed, despite nearly constant yield, the volumetric productivity decreases, gradually with increasing itaconic titers.

A recent study by Hosseinpour et al. reported 220 g/L IA titer with engineered *Ustilago maydis* cells when the strain is used in a fermentation with in situ product crystallization with $CaCO_3$ [54]. Such an approach focuses to lift the weak acid inhibition at the expense of solids accumulating in fermentation vessel. Our work focuses on upstream fermentation optimization for IA production, without any additional separation process. The findings can further be combined with further separation techniques, e.g. in situ product removal techniques [55, 56].

The observed change in cell morphology is in line with previous observations [40] with *U. maydis* cells and is likely due to the stress-induced formation of conjugation tubes [26]. It should be noted that in its haploid form, *Ustilago maydis* grows like yeast, yet when mating with compatible yeasts, each forms a conjugation tube, to form a pathogenic dikaryotic mycelia, in particular under pH stress [57].

Following this, to understand more the changes in the cell, we quantified the elemental composition of biomass during the itaconic acid fermentation and showed that the carbon content increases by up to 50% during the itaconate production phase, when compared to exponential growth phase, attributed to lipid accumulation.

The desired form of organic acids for further downstream processing is the undissociated acid form, which is predominant at low pH (pKa1 = 3.81, pKa2 = 5.45, Supplementary Figure S1, [58]). This undissociated form generates weak acid stress on the cells, it can diffuse through the membrane into the cell, where it dissociates and causes intracellular acidification [51]. The cytosolic acidification changes the kinetics of enzyme-catalyzed reactions, and, therefore, cell needs to secrete the excess protons, at the expense of ATP in order to maintain pH homeostasis [59]. The effect of extracellular pH on the growth and production of *U. maydis* cells has been quantitatively analyzed in terms of fermentation profile as well as estimates of maintenance energy requirements.

It was thus far often presumed that *U. maydis* does not produce itaconate below a pH of 5. This presumption was mainly based on wild-type data of shaken cultures with limiting concentrations of MES buffer [17, 35], where wt *U. maydis* stopped acid production once the pH dropped below 5. In optimized strains of *U. maydis* MB215, this lower limit of acid production is reduced to as far as 3.5, likely though an increase in the specific production rate. It should be noted that, as observed above for the base

case, these scaled up culture produced significantly higher itaconate titers than previously obtained in smaller scale bioreactors. Even with identical cultures conditions, a scale-down to 1.6 L, performed in a different lab, yielded only 11 g/L of itaconate at a pH of 4 (Supplementary Figure S2), compared to 68 g/L at 16 L scale. The reason for this large difference may be the abovementioned scale-up effects on oxygen supply, or subtle differences in medium components, which will be the subject of further study.

An interesting observation was on nitrogen and phosphate levels after the second glucose pulse, while biomass level is observed to be constant. Speculating that the constant level of biomass is the result of continuous growth and lysis, the energy drain particularly in the second half of the fermentation can be attributed to growth-associated maintenance, in addition to the increased non-growth associated maintenance due to weak acid stress. Overall, this work is a stepstone in characterizing fermentation performance of a promising itaconic producing host *U. maydis* under industrially relevant conditions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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