High level production of itaconic acid 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 10L scale pH and temperature controlled fermentation, in fed-batch mode. The effect of pH as well as successive rounds of feeding has been quantified via elemental balances. 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 production phase. Taken together, these results strongly illustrate the potential of engineered *Ustilago maydis* in itaconate production at commercial levels.

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, succinic, and adipic at both academic, and industrial studies [1].

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 [2-4]. Various other bacterial or fungal hosts (e.g. *Mannheimia succinoproducens, Aspergillus niger*) are also used for production of organic acids [5-9]. 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.

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 [14, 15]. 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 [16].

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 medium impurities [12, 17]. Alternative production hosts have been identified, e.g., *Pseudozyma*, *Candida* and *Ustilago* strains [18, 19].

Ustilago strains have been shown to be a good candidate 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 [13, 16]. *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 a yeast-like morphology, high stress resistance and insensitivity to medium impurities [10-13]. Its genome (~20Mb) has been recently published ([10], 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 [12]. 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 [20, 21]. Secondary growth continues also after nitrogen depletion, characterized by intracellular lipid formation leading to swollen cells, and the use of intracellular nitrogen pools [16]. Under nitrogen limitation, cell composition changes and hydrocarbons accumulate, leading to higher C/N ratios with larger cell sizes [12, 16, 22]. Osmolarity causes delayed exponential growth phase and decreased maximum oxygen transfer rate, so decreased growth in high osmolarity might accelerate IA secretion [12]. 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 [23] [12].

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 [14]. The itaconate production pathway, and associated genes, were characterized in this strain [22]. 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* [24], enabling the production of up to 63 g/l itaconate at benchtop scale. Further optimization targets include the MttA mitochondrial transporter from *A. terreus* (REF), 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* (REF).

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. 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 [25]. Considering this, the aim of this work is 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* [23] cells using glucose as the carbon source. The fermentation characteristics of the cells has 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

Wildtype *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. [26, 27]. The strains were stored at – 80 ^{0}C in 80% (v/v) glycerol stocks. Stock cultures were grown on YEPS medium consisted 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

Precultivation

Cells, from glycerol stocks, were activated on YEPS agar plates for 24 h at 30 ^{0}C. 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 at 30 ^{0}C for 24 h. Afterwards, Modified Tabuchi Medium (MTM) [13]; consisting of 30 g/L glucose, 1.6 g/L NH_4Cl, 0.5 g/L KH_2PO_4, 0.41 g/L $MgSO_4.7H_2O$, 1 g/L yeast extract, 0.14 g/L $FeSO_4.7H_2O$ was inoculated with the YEPS grown cells. To inoculate the 8 L bioreactor broth, precultures were grown at 30 ^{0}C for 24 h.

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 $MgSO_4.7H_2O$, 0.01 $FeSO_4.7H_2O$, 0.5 g/L KH_2PO_4, 1 mL/L vitamin solution and 1 mL/L trace element solution [27]. Vitamin and trace element solutions were prepared as previously described by Geiser et al. [16]. 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 in 16-L stainless steel stirred tank bioreactor (NLF22, Bioengineering, Switzerland) were conducted at 30 ^{0}C with a working volume of 8 L. 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. pH was adjusted by automatic addition of 10 M NaOH (Merck, Germany) to pH6 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 CDW and cell composition

At selected time intervals, fermentation broth was centrifuged at 10000 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^{o}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 ^{0}C 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 [28]. Phosphate contents were analysed 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:

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

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.

*Vitamin solution: 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

**Trace element solution: 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, 0.01 g/L KI

Results and Discussion

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 TKN decrease in the first 48 hours, 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 similar for both strains, while WT strain produces considerable amount of mixed organic acids, the mutant strain produce itaconic acid virtually without byproducts detectable by HPLC (Figure 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 and following Geiser et al, [27] the peak is attributed to 2-hydroxyparaconate and values should be taken as rough estimates only.

Figure 1: Comparison of the fermentation and itaconic acid production profile for the wild type (dashed lines in all plots) and mutant (solid 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, 2HP: 2- hydroxyparaconate. The mutant has no significant acid production other than itaconic acid. Shown are representative data from single fermentations.*

We compare the obtained fermentation performance with earlier works, whereby *A. terreus* is used for itaconic acid production at (near-)commercial levels [29-31] (Table 1). Taken together, significant levels of itaconic acid are produced using this scaled up fermentation process in a relatively short fermentation time with high volumetric productivity. Surprisingly, this is also the case compared to previously published *U. maydis MB215 Δcyp3* P_{etef}*ria1* fermentations at benchtop scale [27]. 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 (REF)

Fermentation strategy: feeding time, number of feeding cycles

The following experiments are 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 5 feeding cycles.

Figure 2: Representative data on fed-batch cultivation of itaconic acid producing U. maydis MB215 Δcyp3 P_{etef}*ria1, using pulse feeding. Glucose (circles), itaconic acid (squares), biomass (diamonds) are presented in g/L and culture volume in L (dashed line).*

Glucose was pulsed 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 glucose consumption as well as itaconate production rates of the *U. maydis* MB215 Δ*cyp3* P_{etef}*ria1* cells in each feeding cycle are important parameters, since they allow to decide whether successive pulse feeding is possible, with the same biomass. 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. Based on this data, the base case scenario is set to 2 feeding cycles, corresponding to approximately 48^{th} and 96^{th} 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 2 pulse feeding cycles, the fermentation profile including the offline and online data is given in Figure 3. Overall, by using the described fermentation strategy, a commercially relevant itaconic acid level (80-90 g/L) is reached within 150 hours with two feeding cycles. Furthermore, the strain produces no other significant organic acids. Biomass is produced in the first 50 hours (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 hours (Figure 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%.

Figure 3: Representative fermentation data 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.

Biomass composition:

Itaconic acid fermentation with *U. maydis* consists of two distinct stages: (i) biomass growth and itaconic production and (ii) 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. This is in line with previous observations [27], and is likely due to the stress-induced formation of conjugation tubes [10]. Additionally, the TKN measurements over time yielded on average a lower nitrogen content than what can be expected from an average biomass composition ($CH_{1.65}O_{0.54}N_{0.14}$ [32]). 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 (Figure 4). When the cells cease secondary growth, exhibited by relatively constant CDW measurements (IA production only, later than 48^{th} 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 already been pointed by von Stockar and Liu, where algea typically storing oils contain considerably less nitrogen than average yeast [32]. Deducing from elemental composition, the reducing power of biomass increases, due to increased carbon content, pointing to lipid accumulation within biomass, in line with previous studies with *Ustilago maydis* under nitrogen starvation conditions [33-35].

Figure 4: Changes in the elemental composition of U. maydis 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.*

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

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, Figure 5, [36]). 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 (REF). 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 [37]. 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 (Figure 6). The carbon- and degree of reduction balances are also reported (Figure 7A). 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 [33, 34].

Figure 5: Theoretical partitioning of itaconic acid and itaconate as a function of pH. At pH 4, 40% of the acid is in undissociated form (H_2ITA), while at pH 6; only 0.15% is in undissociated form.

The general trend is that, as pH decreases, *U. maydis* cells grow slower and produce less itaconic acid (Figure 6), in line with several previous reports with different hosts. In the experiments reported here, the biomass level after 48 hours 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 (Figure 5), the change being statistically significant considering the batch-to-batch difference as well ($p = 5.4\ 10^{-4}$).

Figure 6: The effect of extracellular pH on the growth and Itaconic acid production. 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 acid production rates. In both panels, the extracellular pH is kept at 6,5,4 and 3.5 (blue, black, red and green lines respectively). 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.

Overall, as the pH decreases, a decrease in yields of biomass and itaconic acid is observed (Figure 7A), until pH 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 (Figure 7). 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.

It was thus far often presumed that *U. maydis* doesn't produce itaconate below a pH of 5. This presumption was mainly based on wildtype data of shaken cultures with limiting concentrations of MES buffer (REF), 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 (REF). 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 (supplemental figure), 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.

Figure 7: *Probing the response to weak acid stress in Ustilago maydis cells.* **A)** *Carbon and Degrees of Reduction recoveries for the corresponding fermentations at different extracellular pH. The relative contributions of biomass, itaconic acid, carbon dioxide and oxygen (black, white, blue and red bars respectively) for each balance is given.* **B***) offgas data from U maydis fermentations, at different extracellular pH conditions.*

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 - 150 g/L [14], while a nitrogen, not phosphate, limitation induces itaconate production in *U. maydis* (REF). Indeed, at the initial stage of the fermentations with *U. maydis* MB215 Δ*cyp3* P_{etef}*ria1* (first 24 hours), the cells mainly grow while nitrogen levels decrease, followed by the production phase where mainly itaconic acid is produced (Figure 1 and 3). Interestingly, 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 happen in *U. maydis* cultures with similar conditions (REF) (Figure 8). From thereon, despite no apparent nutrient limitation, the cells continue itaconic acid production, while keeping the biomass level 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. Lastly, 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 [38, 39].

Figure 8: **A-B***: Dynamics of Nitrogen and Phosphate levels for different extracellular pH levels. Approximately after the second glucose pulse, the culture is not nutrient limited anymore.* ***C-D****: Alkalinity and Osmotic pressure as a function of extracellular pH. In both panels, the extracellular pH is kept at 6,5,4 and 3.5 (blue, black, red and green lines respectively). 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.*

Conclusions

In this work, we reported the production of over 100 g/L itaconic acid, using *U. maydis* MB215 Δ*cyp3* P_{etef}*ria1* in pulse fed-batch mode. Additionally we quantified the change in biomass composition 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. Furthermore, 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. 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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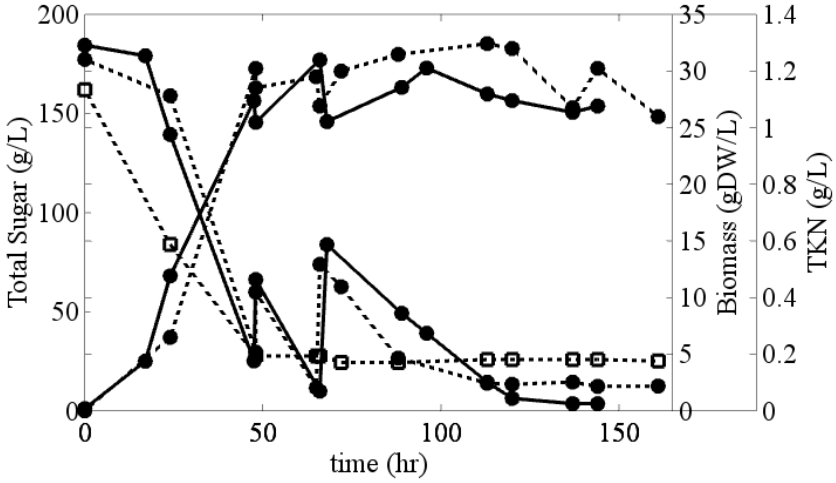
Figures:

A



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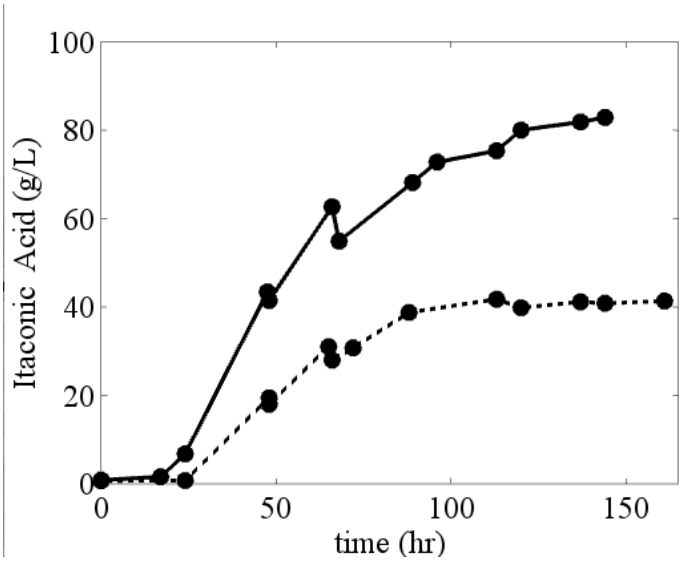

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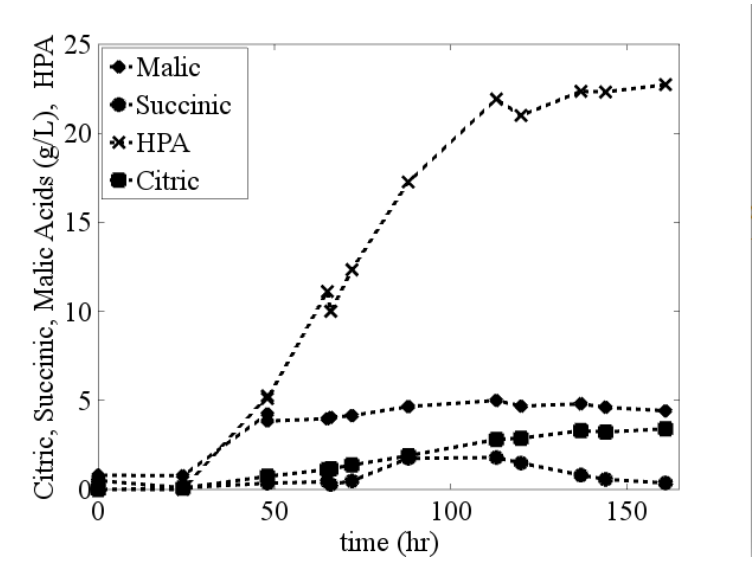

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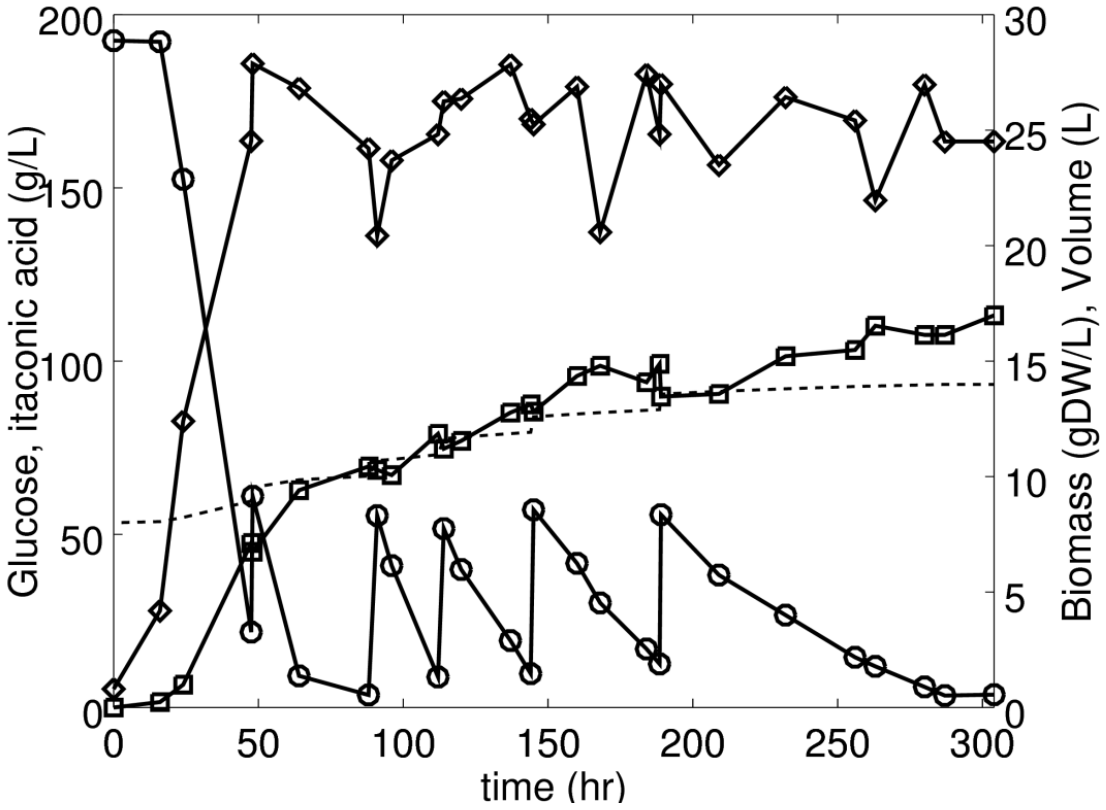

Figure 2: Representative data on fed-batch cultivation of U. maydis MB215 Δcyp3 P_{etef}ria1 at a pH controlled at 6, using pulse feeding. Glucose (circles), itaconic acid (squares), biomass (diamonds) are presented in g/L and culture volume in L (dashed line). Shown are representative data from single fermentations.

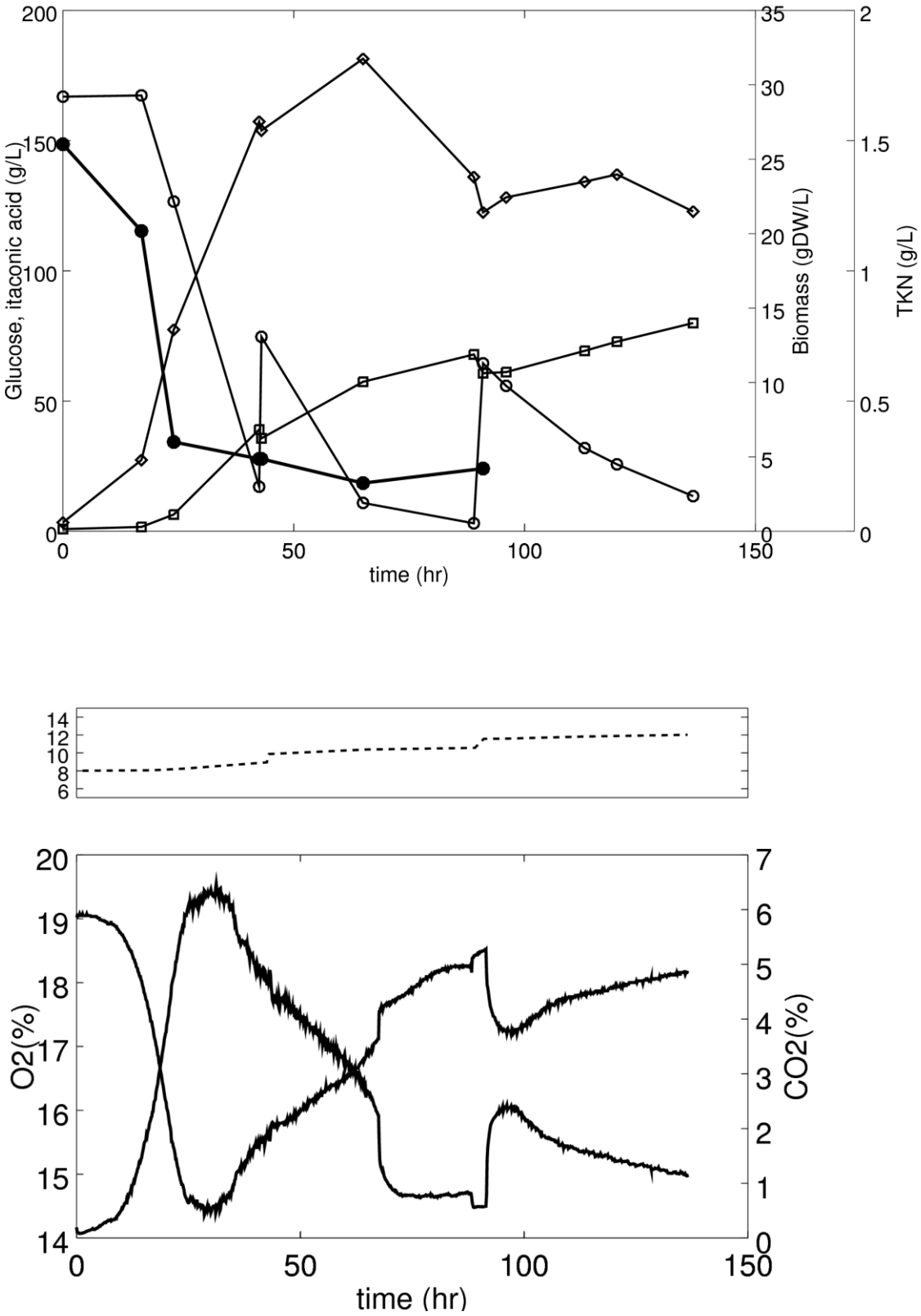

Figure 3: Representative fermentation data on fed-batch cultivation of U. maydis MB215 Δcyp3 P_{etef}ria1 for the base case scenario (pH 6, feeding with two successive substrate pulses) as (a) glucose (circles), itaconic acid (squares), biomass (diamonds) in g/L and TKN (filled circles) in g/L and (b) 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.

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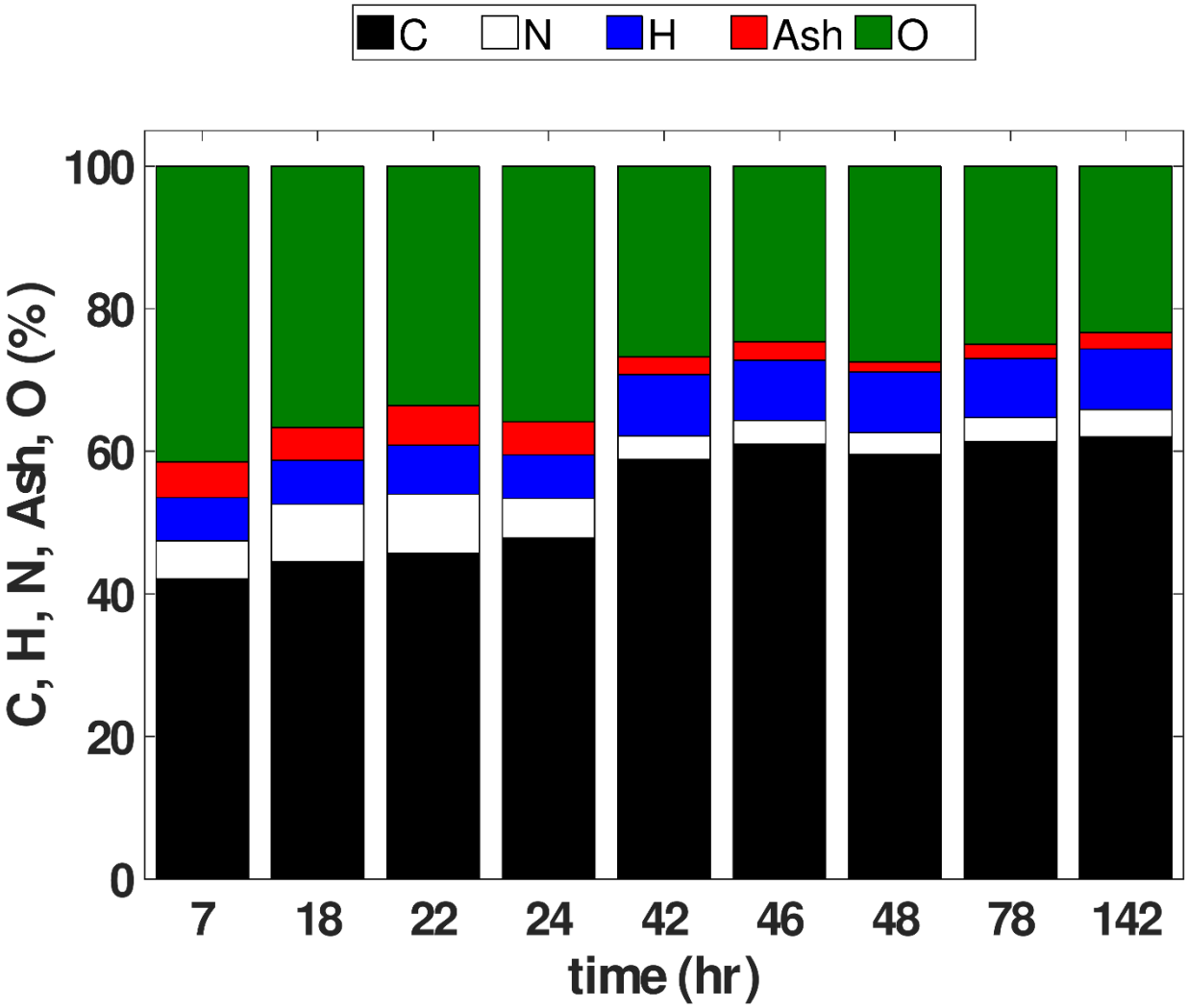

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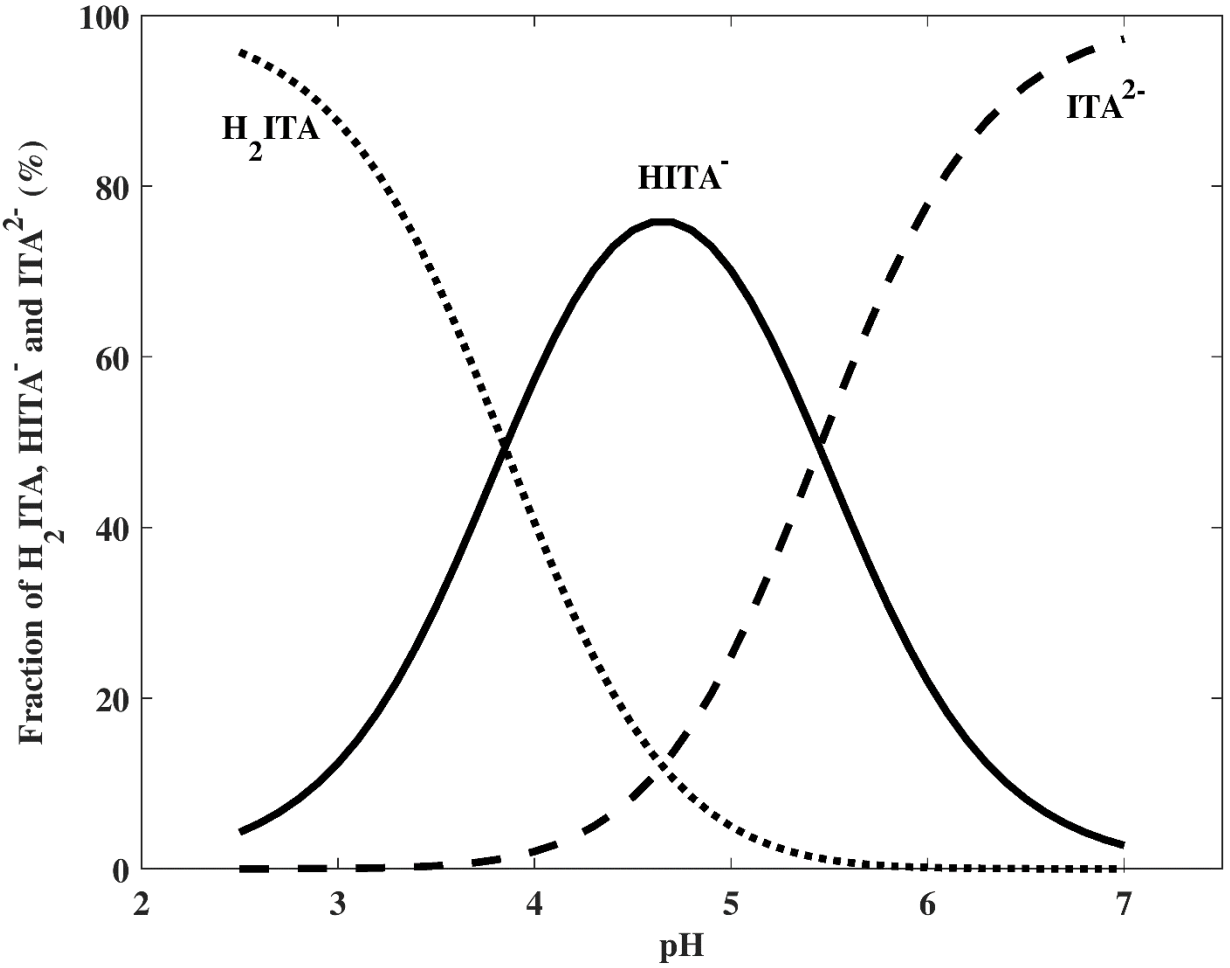

Figure 5: Theoretical partitioning of itaconic acid and itaconate as a function of pH. At pH 4, 40% of the acid is in undissociated form (H_2ITA), while in pH 6; only 0.15% is in undissociated form.

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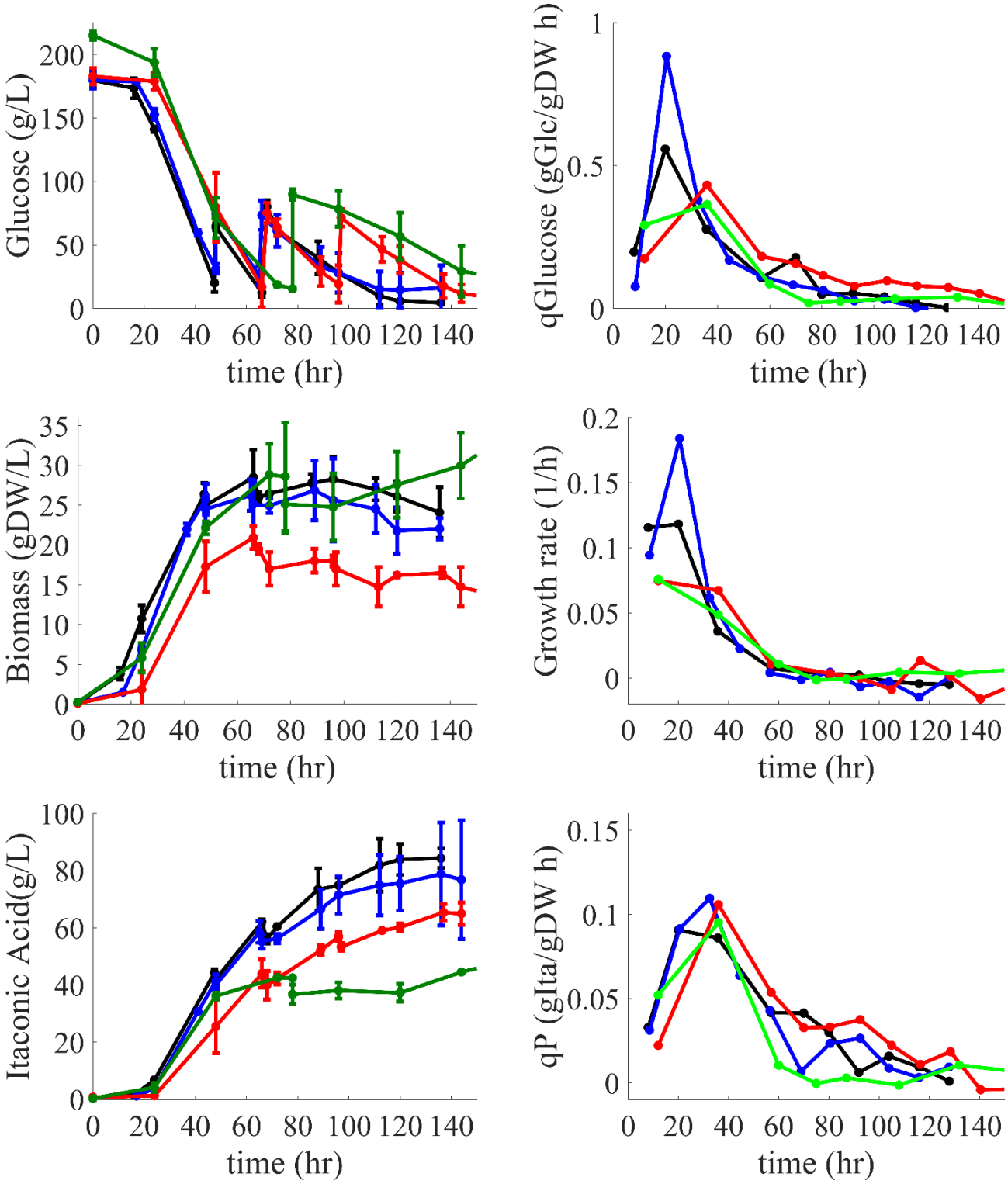

Figure 6: The effect of extracellular pH on the growth and itaconic acid production of U. maydis MB215 Δcyp3 P_{etef}ria1. Left panel describes the measured concentrations of glucose, biomass and itaconic acid while the right panel depics the calculated biomass specific substrate consumption, growth and acid production rates. In both panels, the extracellular pH was kept at 6,5,4 and 3.5 (blue, black, red and green lines respectively). 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 …plicates, error bars depict the deviation from the mean.

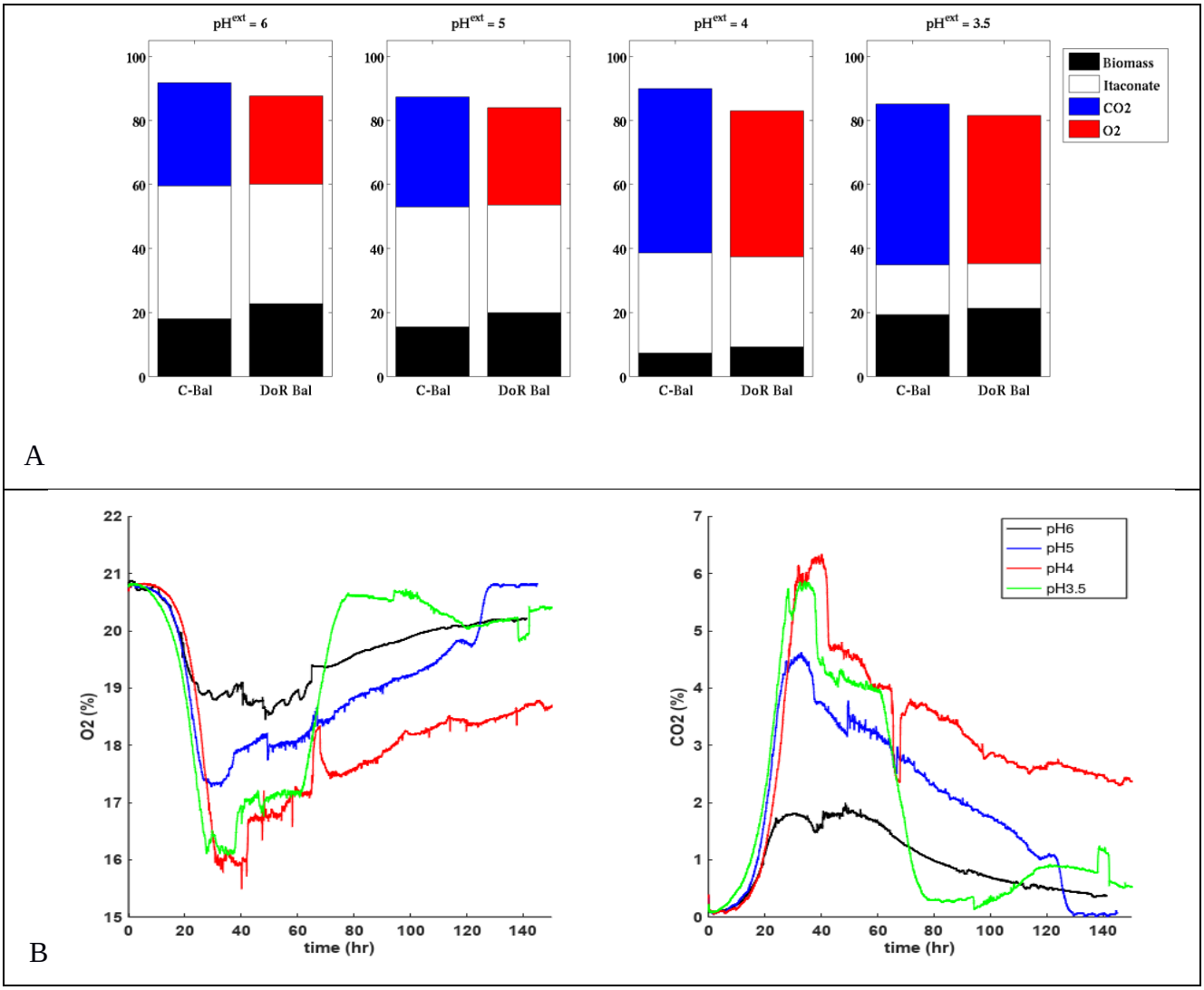

Figure 7: Probing the response to weak acid stress in U. maydis. ***A)*** *Carbon and Degrees of Reduction recoveries for the corresponding fermentations at different extracellular pH. The relative contributions of biomass, itaconic acid, carbon dioxide and oxygen (black, white, blue and red bars respectively) 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 figure 6.*

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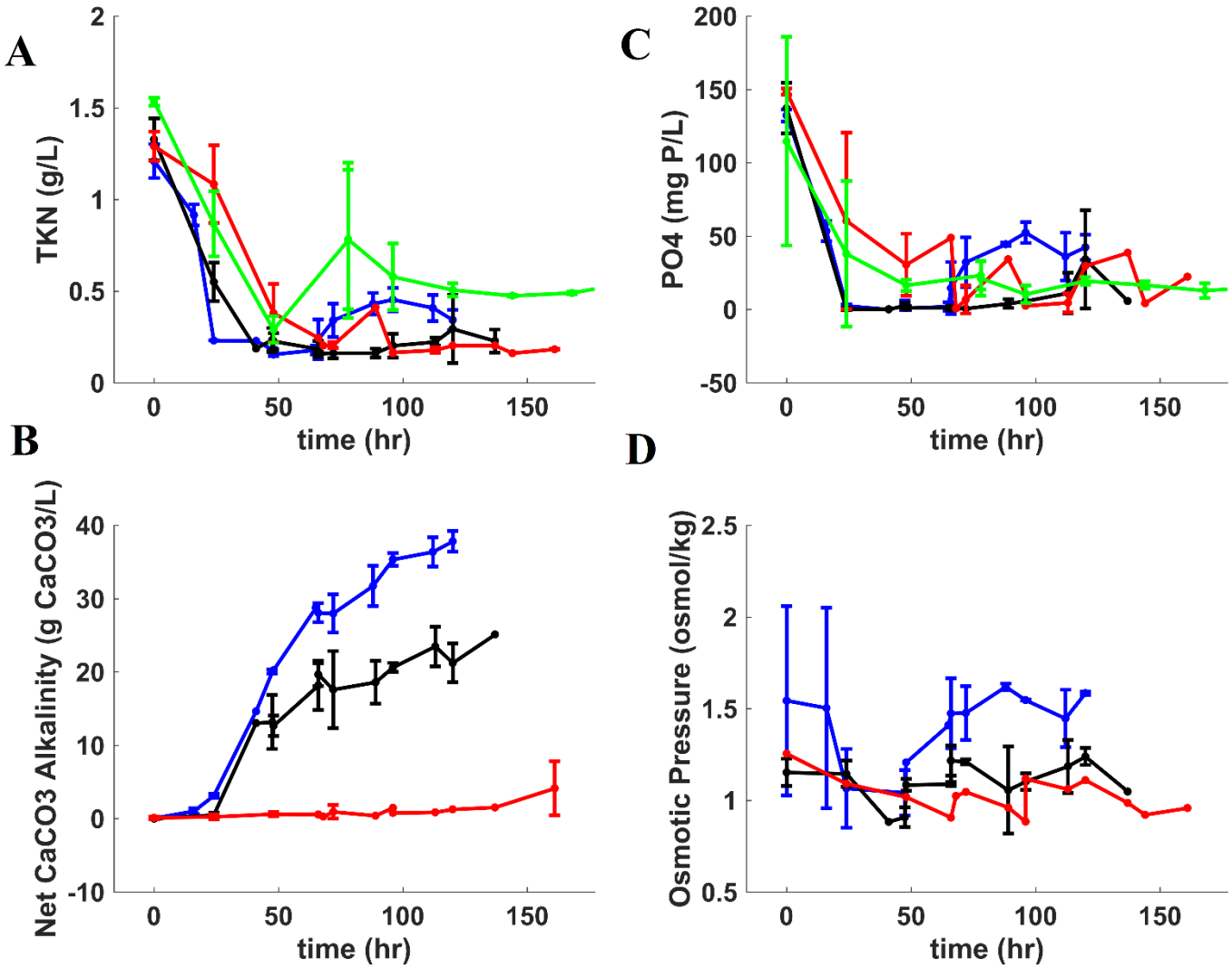

Figure 8: **A-B***: Dynamics of Nitrogen and Phosphate levels for different extracellular pH levels. Approximately after the second glucose pulse, the culture is not nutrient limited anymore.* ***C-D****: Alkalinity and Osmotic pressure as a function of extracellular pH. In both panels, the extracellular pH is kept at 6,5,4 and 3.5 (blue, black, red and green lines respectively). 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 …plicates, error bars depict the deviation from the mean.*

Tables:

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. [29]	*A. terreus DSM-23081*	86.2	0.51	1.2	0.62	7
Hevekerl et al. [30]	*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. [31]	*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., [27]	*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

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Suppelemental data:

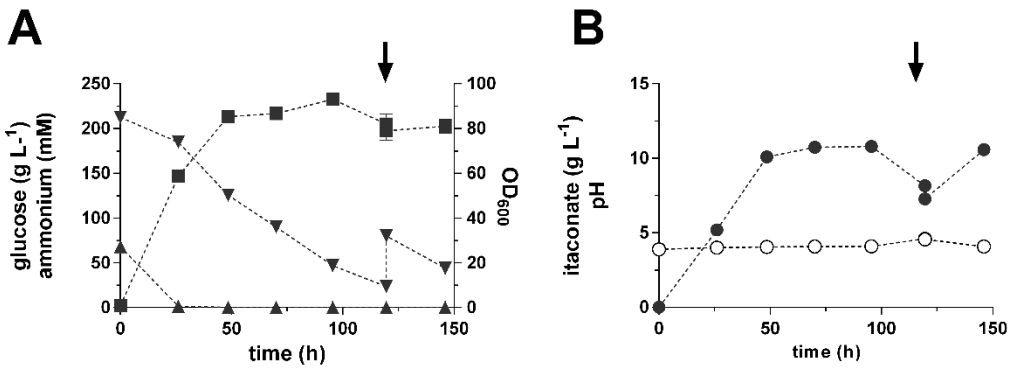

Figure S1. Pulsed fed-batch fermentation of *U. maydis* MB215 Δ*cyp3* P_{etef}*ria1* in controlled bioreactors at 1.6 L scale in production medium with 200 g/l glucose and 4 g/l NH_4Cl. The pH was controlled 4 through automatic addition of NaOH. Air was sparged at a rate of 1 vvm. (**A**) Glucose (▼) and ammonium (▲) concentrations and OD600 (■), (**B**) Itaconate concentration (●) and pH (○). The pH was measured in samples on an external calibrated pH meter. The arrows indicate the addition of a glucose pulse. Cultures were performed in triplicate, error bars represent the standard error of the mean.