New BIOTECHNOLOGY 77 (2023) 30–39

Contents lists available at ScienceDirect

New BIOTECHNOLOGY

journal homepage: www.elsevier.com/locate/nbt

Introducing molasses as an alternative feedstock into itaconate production using *Ustilago sp*

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ARTICLE INFO

Keywords:
Itaconic acid
Alternative feedstocks
Ustilago maydis
Ustilago cynodontis
Process optimisation
Invertase

ABSTRACT

In this work, we established an efficient process for the production of itaconate from the regionally sourced industrial side-stream molasses using *Ustilago cynodontis* and *Ustilago maydis.* While being relatively cheap and more environmentally friendly than refined sugars, there are some major challenges to overcome when working with molasses. Some of those challenges are a high nitrogen load, unknown impurities in the feedstock, and high amounts of ill-favoured carbon sources, such as sucrose or lactate. We could show that the activity of the sucrose-hydrolysing enzyme invertase plays a crucial role in the efficiency of the process and that the fructose utilisation differs between the two strains used in this work. Thus, with a higher invertase activity, the ability to convert fructose into the desired product itaconate, and an overall higher tolerance towards undesired substances in molasses, *U. maydis* is better equipped for the process on the alternative feedstock molasses than *U. cynodontis.* The established process with *U. maydis* reached competitive yields of up to 0.38 g g^{-1} and a titre of more than 37 g L^{-1}. This shows that an efficient and cost-effective itaconate production process is generally feasible using *U. maydis,* which has the potential to greatly increase the sustainability of industrial itaconate production.

1. Introduction

The ever-increasing, worldwide demand for sustainable products is a promising gateway to the development of new bio-based processes. One strategy to make a process more sustainable is by omitting the use of fossil-based feedstocks and instead choosing a feedstock from a renewable source, such as industrial by-products or waste streams. However, alternative carbon feedstocks come with their own set of challenges, such as impurities, the necessity of pre-treatment or limited usability in bulk processes due to availability issues. Nevertheless, several processes could already demonstrate that economically viable processes are possible using renewable resources.

One group of chemicals in which the shift to bio-based production has been successfully demonstrated are organic acids, e.g. citric or lactic acids, which are already being produced through fermentation on a large, industrial scale, partially using industrial waste-products such as sugarcane by-products or starch-based feedstocks as the sole carbon source [1,2]. Another promising candidate in the group of organic acids is itaconic acid, which is widely recognised as a platform chemical and therefore has a significant industrial relevance with an annual market of US$86.8 M in 2018 and an expected rise to US$117.1 M in 2026 [3]. Since itaconic acid can be converted into a wide array of derivatives such as poly(acrylamide-co-itaconic acid) which is marketed as a superabsorbent hydrogel [4], or poly(acrylonitrile-co-itaconic acid), which has high electric conductivity [5], itaconic acid serves a plethora of possible applications.

Today, the industrial production of itaconate is carried out using the fungus *Aspergillus terreus*, because of its high tolerance towards low pH as well as high titres and yields [6]. However, *A. terreus* is a pathogenic producer strain, so that its use is highly regulated within the EU [7], making the production more cost-intensive and laborious. As well as the governmental regulations for pathogenic strains in many countries, the production of itaconate using *A. terreus* comes with further disadvantages, as the filamentous growth of the fungus leads to reduced oxygen

Abbreviations: MTM, modified Tabuchi medium; YEPS, yeast extract, peptone, sucrose medium; GC-ToF-MS, gas chromatography coupled to time-of-flight mass spectrometry; ITA, itaconate; OTR, oxygen transfer rate; CTR, carbon dioxide transfer rate; CDW, cell dry weight; DO, dissolved oxygen.

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https://doi.org/10.1016/j.nbt.2023.06.003
Received 20 January 2023; Received in revised form 15 June 2023; Accepted 16 June 2023
Available online 17 June 2023

transfer and susceptibility to shear stress [8]. To overcome these challenges, alternative organisms for the production of itaconate, such as *Escherichia coli*, *Candida sp.*, or *Ustilago sp.* have been explored, especially within the past decade [9–11].

The basidiomycete *Ustilago sp.* is particularly promising, mainly by virtue of the yeast-like morphology of some of its candidates and its natural ability to produce itaconate [12]. Two *Ustilago* strains have been the subject of several studies addressing itaconate production, *U. maydis* and *U. cynodontis*. Extensive metabolic engineering has been conducted, resulting in two highly optimised strains that produce high amounts of itaconate without unwanted side products while maintaining their yeast-like morphology throughout the process [13,14]. The two strains enable an economically viable process with maximum titres of 83 g L^{-1} and 220 g L^{-1} and yields of 0.61 g g^{-1} and 0.39 g g^{-1} for *U. cynodontis* and *U. maydis*, respectively [15,16]. These results compare highly to those achieved in the conventional production using *A. terreus* where for example 160 g L^{-1} itaconate with a yield of 0.56 g g^{-1} were reached [17]. Thus, *Ustilago sp.* are regarded as realistic alternative producer strains for the bio-based production of itaconate in Europe or other parts of the world.

The above-mentioned titres for *Ustilago sp.* were reached in processes utilising commercial glucose as the sole carbon source. However, the sheer number of publications addressing the issue of alternative feedstocks reveals an increased awareness for making bio-based processes even more sustainable. So far, there have been a few studies addressing this topic for the production of itaconate with *Ustilago sp.*, introducing lignocellulosic feedstocks such as beech wood or brewer's spent grain, which reached itaconate titres of up to 55 g L^{-1} and yields of 0.48 g g^{-1} on beech wood [8,18,19]. However, the necessity to pre-treat the raw materials to obtain sugars for fermentation, thus resulting in a more extensive and costly process, is a significant downside to lignocellulosic feedstocks.

Another industrially relevant feedstock of interest is molasses. This by-product of the sugar industry contains large amounts of readily available sugars and is available in liquid form, and thus can be used directly in fermentation. Further advantages of molasses include its relatively low price and regional availability with 88 sugar manufacturers dispersed over the EU alone [20]. In the past, the fermentation of molasses has been studied for a multitude of organisms and products and is widely used in the production of citric acid, alcohols, and single-cell proteins [21–23]. Although the organic compounds and minerals found in molasses are beneficial for some processes, some components might also exert an inhibitory effect on certain organisms. Molasses used for citric acid fermentation must be pre-treated to remove trace elements since the producing organism *Aspergillus niger* is sensitive to these components [24].

The high amount of organic and inorganic compounds found in molasses is also a challenge in producing itaconate with *Ustilago sp.* It is well known that the presence of nitrogen sources in the medium leads to the growth of the organism while itaconate production is inhibited. Production is activated when the nitrogen sources are depleted [18]. Therefore, the medium must have a suitable C/N ratio to ensure a proper growth phase without restricting itaconate synthesis in the subsequent production phase. Another challenge faced when using molasses in fermentation is the available carbon source. With roughly 65% of the total measured components, sucrose is the main component of molasses (Fig. 1). While understanding the mechanism by which sucrose is utilised by the organism can be crucial to optimising the fermentation, to our knowledge there are no studies so far addressing sucrose utilisation by *Ustilago sp.*

In this study, the composition of regionally supplied beet molasses was investigated, focusing on process-relevant compounds such as nitrogen and carbon sources. Additionally, the influence of the high nitrogen load found in molasses on the process was examined, as well as the mechanism by which sucrose is consumed by *Ustilago sp. U. cynodontis* and *U. maydis* were compared in terms of efficiency and overall titre in fermentations with molasses as the carbon source.

2. Materials and methods

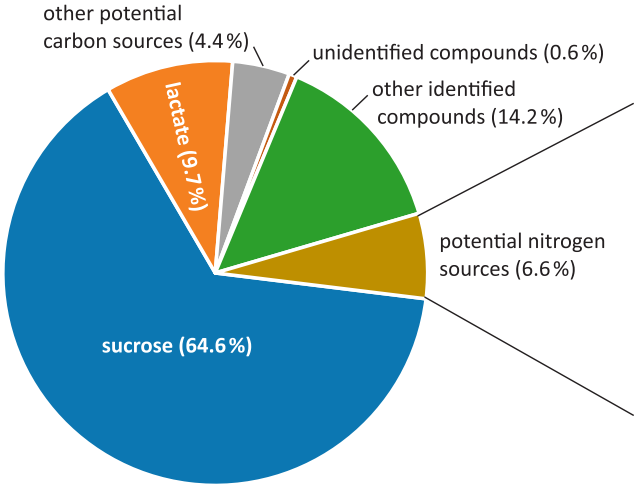
2.1. Chemicals and strains

All chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA), Thermo Fisher Scientific (Waltham, MA, USA), Alfa Aesar (Haverhill, MA, USA), or VWR (Radnor, PA, USA) and were of analytical grade.

Ustilago cynodontis Δ*fuz7*$_r$ Δ*cyp3*$_r$ P$_{etef}$*mttA* P$_{ria1}$*ria1* and *Ustilago maydis* MB215 Δ*cyp3* Δ*MEL* Δ*UA* Δ*dgat* ΔP$_{ria1}$::P$_{etef}$ Δ*fuz7* P$_{etef}$*mttA* [15, 25] were used for this study.

2.2. Microscale cultivations

Pre-cultivations for microscale experiments were performed in 500 mL baffled shake flasks with a filling volume of 50 mL YEPS



component	functional group	amount [%]
glutamine	amino group (-NH_3^+)	2.5
γ-aminobutyrate	amino group (-NH_3^+)	1.7
methoxyamine	amino group (-NH_3^+)	1.3
dimethylglycine	tertiary ammonium group ($(CH_3)_2N^+$)	0.4
glycine	amino group (-NH_3^+)	0.3
aspartate	amino group (-NH_3^+)	0.2
trimethylglycine	quarternary ammonium group ($(CH_3)_3N^+$)	0.1
isoleucine	amino group (-NH_3^+)	0.1

Fig. 1. Composition of beet molasses. Untargeted GC-ToF-MS analysis was performed on a sample of beet molasses received from Pfeifer & Langen company (Jülich, Germany). Components classified under "unidentified" produced peaks in the measurement, which could not be identified with the database at hand. The grouping "other" includes molecules that could be identified but were present in low amounts and/or not relevant to this work. The percentage amount was calculated from the respective proportion of the peak area of a compound in relation to the summed areas of all detectable peaks.

(10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} sucrose). The cultivations took place in an Infors HT Multitron Pro incubator (Infors AG, Bottmingen, Switzerland) at 30 °C and 250 rpm.

All main cultivations were performed in defined modified Tabuchi medium (MTM) adapted from [26]. Changes to the mentioned compositions are described in the Results and Discussion section. The standard composition of the culture medium was as follows: 0.8–6 g L^{-1} NH_4Cl, 0.4 g L^{-1} $MgSO_4$•7 H_2O, 0.01 g L^{-1} $FeSO_4$•7 H_2O, 2 g L^{-1} KH_2PO_4, 0.1 M MES buffer (pH 6.5) and 1 mL L^{-1} trace element solution. The trace element solution contained (per litre) 15 g EDTA, 4.5 g $ZnSO_4$•7 H_2O, 0.84 g $MnCl_2$•2 H_2O, 0.3 g $CoCl_2$•6 H_2O, 0.3 g $CuSO_4$•5 H_2O, 0.4 g Na_2MoO_4•2 H_2O, 4.5 g $CaCl_2$•2 H_2O, 3 g $FeSO_4$•7 H_2O, 1 g H_3BO_3 and 0.1 g KI. Varying carbon sources were supplied to the medium as stated for each experiment. The amount of NH_4Cl differed depending on the amount and type of carbon source used and is specified for each experiment individually.

All microscale experiments were performed in microtiter plates of type MTP-48-BOH1 FlowerPlates® covered with F-GPRSMF32–1 gas-permeable sealing foils (all from Beckman Coulter, Baesweiler, Germany) and working volumes of 800 µL. For cultivation, a BioLector Pro microbioreactor system (Beckman Coulter) was applied at 30 °C, 1400 rpm shaking frequency and relative humidity of ≥ 85 %. Backscatter, pH, and DO were measured every 10 min for each cultivation well. The backscatter measurements were obtained with the gain set to 5. Values attained as such are given in arbitrary units and represent the amount of light scattered back by the cultivation broth. Thus, a higher backscatter value correlates with higher biomass in the well. Sacrifice samples of the cultivations were taken automatically by a Freedom Evo 200 robotic platform (Tecan Group, Männedorf, Switzerland), into which the BioLector Pro is integrated [27].

Each cultivation was performed with ≥ 8 replicates for each condition. For the backscatter values, every replicate is represented by a single line graph in the respective figure. Upon sampling, one replicate at a time was sacrificed to ensure a closely sampled dataset. The exact number of replicates per condition for the growth curves can thus be derived from the amount of time-triggered samples drawn per experiment.

The data from the cultivations were analysed with the in-house package *bletl* [28] and visualised using matplotlib version 3.5.2 [29].

2.3. Bioreactor cultivations

For cultivation experiments in stirred tank bioreactors *U. maydis* was plated on YEPS agar plates containing 10 g L^{-1} yeast extract, 10 g L^{-1} peptone, 10 g L^{-1} sucrose and 20 g L^{-1} agar. The culture was grown for two days at 30 °C. Liquid pre-cultures were inoculated from the plate and grown in 500 mL shake flasks with a filling volume of 50 mL MTM as described above containing 1 g L^{-1} NH_4Cl and 1 mL L^{-1} vitamin solution (per litre: 0.05 g D-biotin, 1 g D-calcium pantothenate, 1 g nicotinic acid, 25 g myoinositol, 1 g thiamine hydrochloride, 1 g pyridoxal hydrochloride and 0.2 g para-aminobenzoic acid). As a carbon source 50 g L^{-1} sucrose equivalents from molasses were added. Pre-cultures were cultivated for 24 h.

Main cultivations were performed in MTM as described for the pre-cultures but without MES and with an additional 1 g L^{-1} yeast extract. Molasses was used as the sole carbon source in the batch medium as well as the feed.

Fermentations were performed using 2 L Sartorius BIOSTAT® Bplus stirred tank reactors (Sartorius, Goettingen Germany). The fermenters were equipped with one six-blade Rushton turbine and 4 baffles. During the cultivation, the temperature was set to 30 °C. The dissolved oxygen tension (DOT) was measured using a VisiFerm™ DO 225 pO_2 sensor (Hamilton, Hoechst, Germany) and kept ≥ 30 % automatically, firstly by increasing the stirring rate from 800 to 1200 rpm and secondly by adjusting the aeration rate from 1 L min^{-1} to 3 L min^{-1}. The pH was measured using an EasyFerm Plus K8 pH sensor (Hamilton) and controlled at a value of 6.5 by the addition of 5 M NaOH and 1 M HCl. Off-gas analysis was performed using a DASGIP GA4 exhaust gas analyser (DASGIP, Eppendorf, Juelich, Germany). 1 mL of Antifoam 204 was added at the beginning of the cultivations and afterwards when necessary to control foaming. Throughout the cultivation, time samples were taken from the bioreactor for offline analysis. The fermentation started with an initial volume of 1 L. Volume changes due to feeding, pH adjusting agents and sampling were taken into account for the analysis.

2.4. Biomass and supernatant analyses

Cell dry weight (CDW) was determined by weighing the dried cell pellets of three 2 mL samples (centrifuged at 15,000 *g* for 10 min, dried overnight at 80 °C) for each sample point.

Concentrations of relevant metabolites in supernatants were determined by HPLC using an Agilent InfinityLab LC series Infinity II 1260 system (Agilent Technologies, Santa Clara, CA, USA) equipped with a refractive index detector (RID). The different components were separated using an organic acid resin column (Chromatographie Service GmbH, Langerwehe, Germany) heated to 30 °C with a mobile phase of 10 mM H_2SO_4 running at 0.6 mL min^{-1}. For the 1 L fermentations a Thermo Fisher Ultimate 3000, (Thermo Fisher Scientific) equipped with an ERC RefrctoMax 520 RID (Shodex, München, Germany) was used. For separation, a ROA-Organic Acid H+ (8 %) (300 × 7.8 mm) (Phenomenex, Torrance, CA, USA) was heated to 30 °C and used with a mobile phase of 5 mM H_2SO_4 running at 0.8 mL min^{-1}.

2.5. Invertase activity assay

Invertase activity was measured according to a protocol first performed on *Saccharomyces cerevisiae* [30]. Pre-cultures of the strains were conducted according to the protocol for microbioreactor cultivations. When the early exponential phase of the cultivations was reached, approximately 10^6 cells were harvested, washed 3 times with sterile ddH_2O and resuspended in $YPD_{0.05}$ (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 0.5 g L^{-1} glucose) to an OD_{600} of 0.3. The cultures were incubated further for 2 h, while samples were taken every 20 min, washed twice with sterile ddH_2O and kept on ice until further processing. After 2 h, all samples were resuspended in 50 µL 50 mM Na acetate (pH 5.1). To start the conversion reaction, 6.25 µM sucrose was added to each sample for an incubation time of exactly 10 min at 30 °C. The reaction was stopped by adding 75 µL 0.2 M K_2HPO_4, placing the samples on ice for 1 min, boiling them for 3 min, and placing them on ice again. To start the colour reaction, 500 µL assay mix (250 U glucose oxidase, 62.5 µg peroxidase, 3.75 mg o-dianisidine in 25 mL 0.1 M KH_2PO_4 pH 7.0) was added to the samples before incubating them for 20–30 min at 37 °C. After the incubation, 500 µL 6 M HCl was added to develop the colour and the samples were measured at 540 nm. To calculate the amount of glucose formed in the assay, an external standard containing 250 µM glucose was subjected to the assay together with the samples. The invertase activity is expressed through the amount of glucose [µM] formed per minute per 10^6 cells.

2.6. Untargeted GC-ToF-MS analyses

Before analysis 13 or 130 µL aliquots of the samples were shock frozen in liquid nitrogen, stored at − 20 °C and then lyophilised overnight in a Christ LT-105 freeze drier (Martin Christ Gefriertrocknungs-Anlagen, Osterode am Harz, Germany). The dried samples were consecutively derivatized with 50 µL MeOX (20 mg mL^{-1} O-methyl hydroxylamine in Pyridine) for 90 min at 30 °C and 600 rpm in an Eppendorf Thermomixer, followed by incubation with an additional 80 µL of MSTFA (N-acetyl-N-(trimethylsilyl)-trifluoroacetamide) for 90 min at 40 °C and 600 rpm. For the determination of the derivatized metabolites an Agilent 8890 N double SSL gas chromatograph (Agilent, Waldbronn, Germany) equipped with an L-PAL3-S15 liquid autosampler

was used, coupled to a LECO GCxGC HRT+ 4D high-resolution time of flight mass spectrometer (LECO, Mönchengladbach, Germany). The system was controlled by the LECO ChromaToF software. 1 µL sample was injected into a split/splitless injector at 280 °C at varying split modes. For 1D GC analysis, the Back Injector was equipped with a 30 m Agilent EZ-Guard VF-5 ms + 10 m guard column (Agilent,). For 2D GCxGC analysis, the Front Injector was equipped with a 30 m HP 5-ms Ui column (HP) connected to a 2 m Rtxi17 (Restek) in a secondary oven. Constant Helium flow was adjusted to 1 mL min^{-1} for the active injector and column and to 0.5 mL min^{-1} for the passive injector. The GC temperature programme starts at 60 °C with a hold time of 2 min, followed by a temperature ramp of + 12 °C min^{-1} up to the final temperature of 300 °C, hold time of 8 min, leading to a total run time of 30 min. The secondary oven temperature offset was set to + 15 °C above the first oven temperature. The transfer line temperature was set to 300 °C. The ToF MS was operated in positive electron impact $[EI]^+$ mode at an electron energy of 70 eV. The ion source temperature was set to 250 °C. The MS was tuned and calibrated with the mass fragmentation pattern of PFTBA (PerFlouroTriButylAmine, Heptacosa). During analysis, the accurate masses were corrected to a single-point lock mass of Heptacosa as an external reference at *m/z* 218.9856. 1D Data acquisition was performed in stick mode with a scan rate of 200 scans s^{-1} To identify known metabolites a baseline noise-corrected fragmentation pattern together with the corresponding current RI value (Retention time Index) was compared to our in-house accurate *m/z* database JuPoD, and the commercial nominal *m/z* database NIST20 (National Institute of Standards and Technology, USA).

Unknown peaks were identified by a virtual reconstruction of the derivatized metabolite structure via the measured baseline noise corrected accurate mass *m/z* fragment pattern in comparison to an accurate *m/z* fragment register inside the JuPoD main library and were subsequently verified by virtual derivatization and fragmentation of the predicted structure.

2.6.1. Molasses composition analyses

Relevant elements and ions in molasses were measured by the ZEA-3 institute of the Forschungszentrum Jülich GmbH. Trace elements in molasses were measured by inductively coupled plasma optical emission spectroscopy. Samples of 250 mg were dissolved in 3 mL HNO_3 + 1 mL H_2O_2 and filled up to a volume of 50 mL with water. The samples were diluted at 1:100 and analysed in an iCAP 7600 device. Ammonia and phosphate were measured via continuous flow analysis in an alliance instruments device (Alliance Instruments GmbH, Salzburg, Austria). 0.5 g samples were diluted in 50 mL H_2O before analysis.

Ion chromatography was performed to measure Cl^-, NO_3-, and SO_4^{2-}. A 1:10 dilution of molasses was analysed using an ICS-3000 device and an AS 14 A column (both Thermo Fisher Scientific,). The eluent used for the procedure contained 1 mM $NaCO_3$ and 8 mM Na_2CO_3.

Elemental analysis was performed on a Vario EL cube system (Elementar Analysensysteme GmbH, Langenselbold, Germany). Samples were prepared as follows: 3 × 2 mg in CHN-mode, 3 × 2 mg in S-mode, and 3 × 2 mg in O-mode.

3. Results and discussion

Due to its regional availability and relatively low price, molasses is a promising alternative to commercially available purified sugars,but as far as we are aware, no studies have been published for *U. cynodontis* or *U. maydis* using molasses as feedstock for the production of itaconate. There have, however, been several studies with molasses as the carbon source for other processes, such as the production of baker's yeast and ethanol using *S. cerevisiae* or lysine with *U. maydis*, which have resulted in viable processes with competitive yields and titres [31–33].

3.1. Beet molasses composition

Molasses contains various organic and inorganic compounds which can affect the itaconic acid production process. To assess the influence of these components on *Ustilago sp.,* the composition of the beet molasses used in this study was analysed. The analysis using a GC-ToF-MS system revealed a sucrose fraction of 64.6 % and a lactate content of 9.7 % (Fig. 1). Other carbon sources, such as malate or mannitol made up only 4.4 % of the measured components in the molasses. As mentioned previously, the most efficient itaconate production with *Ustilago sp.* was achieved with glucose as the sole carbon source. Moreover, the sucrose utilisation of the organism has not been studied yet, making the high sucrose content of the molasses a possible challenge for the fermentation.

Further relevant molecules are those that could potentially serve as a nitrogen source for *Ustilago sp.* since nitrogen limitation induces the production of itaconate in this organism [34]. According to our quantitative analysis, pure molasses contains roughly 40 mM NH_4^+ (Supplementary Table S1). However, additional nitrogen sources such as the amino acids glutamine and aspartate were also found in the molasses (Fig. 1). While it has been reported that glutamine is not favoured over ammonium by *U. maydis,* glutamine pathway-related genes, as well as genes for the utilisation of nitrate are present in the fungus, suggesting that the assimilation of alternative nitrogen sources is possible [35,36].

3.2. Growth and itaconate production of Ustilago sp. at different C/N ratios

As mentioned above, itaconate production in *Ustilago sp.* is initiated by nitrogen limitation and the growth and production phases are largely uncoupled [18,34]. Hence, one key to a successful process on molasses is a suitable C/N ratio to allow for a sufficient growth phase as well as the production of itaconate in the subsequent production phase. Therefore, the optimum NH_4Cl concentration in a medium supplied with 100 g L^{-1} glucose was determined for both *U. maydis* as well as *U. cynodontis.* Previous studies suggest NH_4^+ concentrations of 30 and 75 mM for cultures containing 120 and 200 g L^{-1} glucose, respectively [18,37]. This translates to C/N ratios of 133.2 mol_C mol_N^{-1} and 88.8 mol_C mol_N^{-1} for the media used. Since the cultivation conditions, media composition and strains used in this study differ from those in previous studies, the experiments were repeated for maximum applicability to the process parameters at hand. The best NH_4Cl concentration for both strains was determined to be 45 mM, corresponding to a C/N ratio of 74 mol_C mol_N^{-1} (Supplementary Figs. S1 and S2), which was used in the following experiments with defined media.

3.3. Growth and itaconate production of Ustilago sp. on defined sucrose media

Contributing to the total measured components with a fraction of roughly 65%, sucrose is the main component in molasses (cf. Fig. 1). For a successful production process on the alternative feedstock it is therefore crucial to understand the mechanism with which *Ustilago sp.* metabolise the disaccharide. So far there have been no studies published concerning the sucrose hydrolysis or uptake of *Ustilago sp.* Other organisms such as *S. cerevisiae* or several plant species secrete the enzyme invertase which hydrolyses sucrose outside of the cell [38,39]. According to previous results obtained from fermentations on sucrose, in which glucose and fructose accumulated in the culture broth (data not shown), as well as a prediction for an invertase-encoding gene in the annotated genome of *U. maydis* [26], it was hypothesised that *Ustilago sp.* also carry an active invertase.

To assess the ability of *U. cynodontis* and *U. maydis* to utilise sucrose, both strains were grown in defined MTM containing either 100 g L^{-1} sucrose or glucose as a reference carbon source. Both strains were able to hydrolyse the sucrose completely, which led to an accumulation of

glucose and fructose in the culture broth (Fig. 2). Interestingly, the initial rate at which sucrose was hydrolysed was much higher in *U. maydis*, resulting in an onset of biomass growth as fast as under glucose conditions. In contrast, *U. cynodontis* showed a pronounced lag phase on glucose and even more so on sucrose. Once all glucose was metabolised, the strains started using fructose, albeit at a low rate with almost half of it left in the broth by the end of the fermentation.

Corresponding to the different growth patterns, the production of itaconate varied between the two strains. *U. maydis* started producing itaconate as early as 16 h after inoculation in both glucose- and sucrose-containing medium. Notably, the itaconate titre and yield on sucrose were found to be 26% and 33 % lower compared to the glucose reference (Table 1). For *U. cynodontis* itaconate could be detected after 30 h when grown on glucose, and after 39 h in the medium containing sucrose and the itaconate titre and yield on sucrose were 47 % and 45 % lower compared to glucose.

It should be noted that the measured initial carbon source concentrations for both *U. cynodontis* conditions are below the desired 100 g L^{-1}. This effect of underestimating the sugar concentrations in the medium was observed in several of the experiments presented in this work. We therefore suspect either a systematic analytical error in the form of a matrix effect or a rapid initial uptake of a certain amount of carbon by the *Ustilago* strains due to osmotic factors. Nonetheless, the data allow a comparison of the different conditions, especially within the same experiment.

Table 1
Itaconate production performances of *U. maydis* MB215 Δ*cyp3* ΔMEL ΔUA Δ*dgat* ΔP_{ria1}::P_{etef} Δ*fuz7* P_{etef}*mttA* and *U. cynodontis* Δ*fuz7*$_r$ Δ*cyp3*$_r$ P_{etef}*mttA* P_{ria1}*ria1* in different microscale cultures with glucose or sucrose as the sole carbon source.

Carbon source	*U. maydis*		*U. cynodontis*	
	Titre [g L^{-1}]	Yield[a] [g g^{-1}]	Titre [g L^{-1}]	Yield[a] [g g^{-1}]
100 g L^{-1} glucose	29.14	0.33	23.54	0.33
100 g L^{-1} sucrose	21.46	0.22	12.38	0.18
100 g L^{-1} sucrose (molasses)	0.21	< 0.01	0.07	< 0.01
50 g L^{-1} sucrose (molasses)	14.99	0.30	2.29	0.05
25 g L^{-1} sucrose (molasses)	12.13	0.49	9.47	0.38
75 g L^{-1} sucrose (molasses) + 25 g L^{-1} glucose	28.30	0.34	12.80	0.16
50 g L^{-1} sucrose (molasses) + 50 g L^{-1} glucose	30.42	0.36	13.67	0.16
25 g L^{-1} sucrose (molasses) + 75 g L^{-1} glucose	33.70	0.39	29.36	0.39

[a] Itaconate yield per consumed glucose or sucrose, respectively.

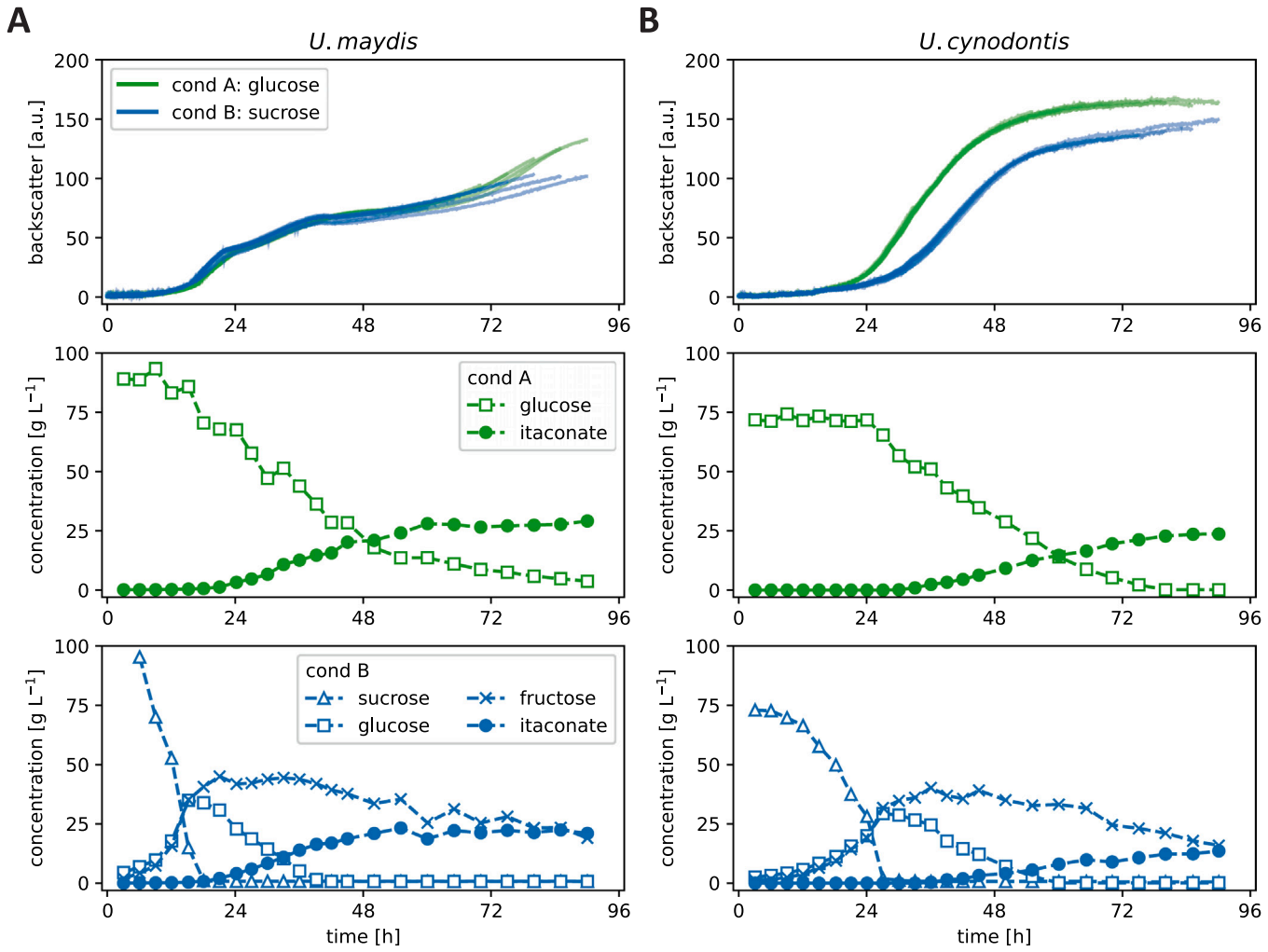


Fig. 2. Growth and itaconate production of A) *U. maydis* MB215 Δ*cyp3* ΔMEL ΔUA Δ*dgat* ΔP_{ria1}::P_{etef} Δ*fuz7* P_{etef}*mttA* and B) *U. cynodontis* Δ*fuz7*$_r$ Δ*cyp3*$_r$ P_{etef}*mttA* P_{ria1}*ria1* in defined MTM containing 100 g L^{-1} glucose (cond A) or sucrose (cond B), respectively. Microscale cultures were performed in a BioLector Pro at 30 °C, 1400 rpm, 85% humidity and 800 µL culture volume. The pH and DO profiles corresponding to this experiment are depicted in Supplementary Fig. S3.

3.4. Invertase activity of Ustilago sp. on defined and molasses media

A reason for the superior growth and production performance of *U. maydis* on sucrose could be a higher invertase activity, which makes glucose available earlier in the process. As postulated above, the presence and functionality of an enzyme exhibiting invertase activity in *Ustilago sp.* could be verified in an invertase activity assay, as previously published for *S. cerevisiae* [30]. The enzyme activity in samples of approximately 10^6 cells was observed for 120 min after the cells were transferred from a medium containing 2 % glucose to a medium with only 0.05% glucose (Fig. 3). Throughout all examined time points, a steady invertase activity was measured for both strains, whereby *U. maydis* was found to have exceedingly higher sucrose-hydrolysing activity compared to *U. cynodontis*.

The invertase activities for *U. maydis* with a maximum of 214 μM min^{-1} (10^6 cells)$^{-1}$ surpassed those reported for *S. cerevisiae* with a maximum of 150 μM min^{-1} (10^6 cells)$^{-1}$, whereas the activity in *U. cynodontis* was significantly lower, averaging at 23.7 μM min^{-1} (10^6 cells)$^{-1}$. Additionally, in *S. cerevisiae*, the presence of glucose inhibits the invertase activity, leading to a steady increase in sucrose-hydrolysing activity for 2 h after the transfer to a medium containing lower amounts of glucose [30]. Thus, the results suggest that for *Ustilago sp.*, the mechanism of invertase regulation is different from that of *S. cerevisiae* with no inhibiting effect of glucose. Furthermore, the levels of sucrose-hydrolysing activity in both strains were nearly unchanged in the presence of 2 % molasses compared to 2 % glucose, suggesting that the offered carbon source does not influence the invertase activity significantly in either of the two strains.

3.5. Growth and itaconate production of Ustilago sp. on defined fructose media

Once sucrose is hydrolysed, the fungus can use the monosaccharides to produce either biomass or the desired product itaconate. Unlike for glucose, there are no studies relating to the fructose metabolism in *Ustilago* and thus, the yield with which *Ustilago sp.* can convert fructose into itaconate is yet unknown.

To gain more insight into this metabolic process, *U. cynodontis* and *U. maydis* were evaluated in microscale cultivations supplied with either 35, 50, or 100 g L^{-1} fructose (Fig. 4). The growth curves show that *U. cynodontis* produces more biomass the more fructose is available. For *U. maydis*, biomass formation on fructose was considerably lower as compared to growth on sucrose or glucose (cf. Fig. 2A). The metabolic analysis via HPLC reveals that there is also a striking difference in itaconate production between the two strains. While *U. maydis* produced nearly 30 g L^{-1} of the product, *U. cynodontis* showed little to no accumulation of the product, even at the highest fructose concentration. It can therefore be concluded, that the two strains utilise fructose for entirely disparate cellular processes. While the gross majority of the sugar in *U. cynodontis* is used for cell growth and division, *U. maydis* is able to use the carbon source to produce the desired product itaconate, which seems to be a contributing factor to the higher titres for *U. maydis* in sucrose-containing medium (cf. Fig. 2).

3.6. Growth and itaconate production of Ustilago sp. on raw molasses media

The previous experiments with both *Ustilago* strains showed that itaconate production is feasible in defined MTM medium with 100 g L^{-1} sucrose and 2.4 g L^{-1} NH_4Cl. When the alternative feedstock molasses was introduced, a re-evaluation of the appropriate C/N ratio was performed because it was not known which of the nitrogen sources in molasses can be assimilated by *Ustilago sp.* In addition, the effect of possible impurities in the molasses on cell growth and itaconate production was investigated.

Thus, the two strains *U. cynodontis* and *U. maydis* were grown in MTM containing molasses diluted to a total sucrose concentration of 25, 50, or 100 g L^{-1}. No nitrogen sources other than the ones naturally present in molasses were provided, thus the C/N ratio was constant throughout all conditions. Generally, the growth on molasses was less reproducible compared to the growth on commercial sugars, probably owed to impurities (Fig. 5). Biomass production was approximately doubled when 50 g L^{-1} sucrose equivalents from molasses were applied compared to 25 g L^{-1}. Yet, when the molasses concentration was increased to 100 g L^{-1} sucrose, the growth was inhibited, as shown by longer lag phases and lower final biomass for both strains. This suggests that the preferred molasses concentration at which uninhibited growth is possible lies below 100 g L^{-1}.

Further information on the effect of high molasses concentrations on cell growth was gained through microscopic imaging (Fig. 5). Both strains exhibit yeast-like growth behaviour in defined media containing 100 g L^{-1} sucrose and in molasses media with up to 50 g L^{-1} sucrose equivalents. Notably, *U. cynodontis* cells tended to aggregate when 50 g L^{-1} sucrose equivalents from molasses were present. When molasses was applied in higher concentrations, *U. cynodontis* cells formed elongated, filamentous structures. From our experience working with this strain under a variety of conditions, this may be a sign of stress.

The maximum itaconate titres for both strains grown on molasses were comparatively low with a maximum of 14.99 g L^{-1} for *U. maydis* in the broth containing 50 g L^{-1} sucrose equivalents from molasses (cf. Table 1). Yet, the yields – especially at lower molasses concentrations – are promising. While *U. maydis* attained a yield of 0.49 g g^{-1}, which was slightly lower than that recorded on glucose, *U. cynodontis* exceeded the value obtained on glucose with 0.38 g g^{-1}.

Calculated based on our GC-ToF-MS measurements, a C/N ratio of 29.3 mol_C mol_N^{-1} was determined for molasses (Supplementary Table S2), which is significantly lower than the optimum for *Ustilago sp.* established on defined media (cf. Supplementary Fig. S1 and S2). Considering this and the fact that the itaconate yields attained in the medium containing the lowest molasses concentration were in a similar

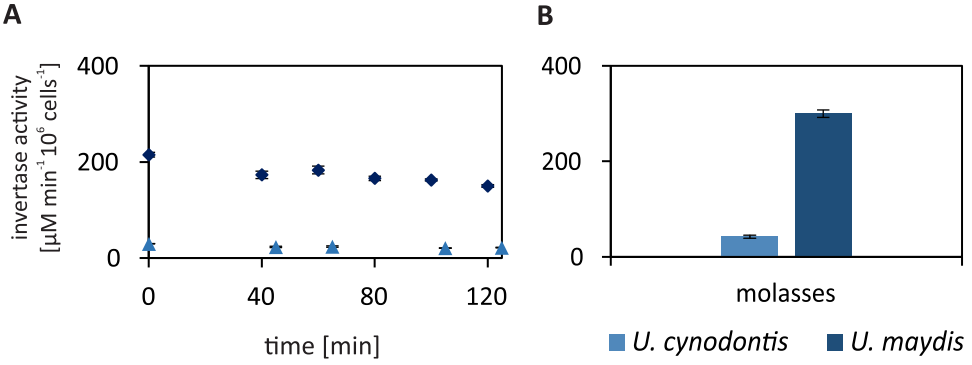


Fig. 3. Sucrose-hydrolysing activity of *Ustilago sp.* as determined by an invertase activity assay. A) Cells of the strains *U. maydis* MB215 Δ*cyp3* ΔMEL ΔUA Δ*dgat* ΔP_{ria1}::P_{etef} Δ*fuz7* P_{etef}*mttA* and *U. cynodontis* Δ*fuz7*$_r$ Δ*cyp3*$_r$ P_{etef}*mttA* P_{ria1}-*ria1* were grown in the presence of 2% glucose until the early exponential phase and then transferred to a medium containing 0.05% glucose for 2 h. Samples of approximately 10^6 cells were taken regularly to determine the invertase activity. B) Invertase activity of cells grown on complex media containing 2% beet molasses. Mean values and standard deviations were derived from three replicates.

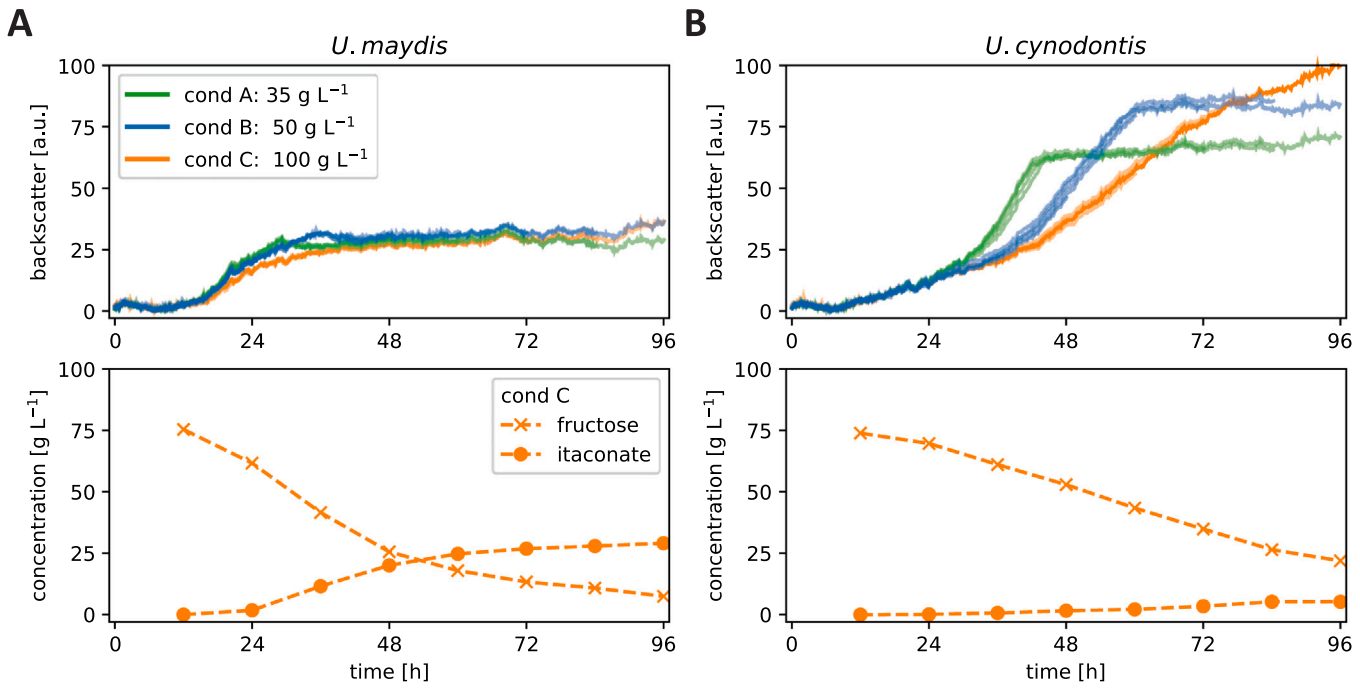


Fig. 4. Growth and itaconate production of A) *U. maydis* MB215 Δ*cyp3* ΔMEL ΔUA Δ*dgat* ΔP_{ria1}::P_{etef} Δ*fuz7* P_{etef}*mttA* and B) *U. cynodontis* Δ*fuz7*$_r$ Δ*cyp3*$_r$ P_{etef}*mttA* P_{ria1}*ria1* in MTM containing varying amounts of fructose as the sole carbon source. The metabolic data shows exemplary itaconate production under 100 g L^{-1} fructose conditions (cond C). Microscale cultures were performed in a BioLector Pro at 30 °C, 1400 rpm, 85% humidity and 800 µL culture volume. The pH and DO profiles corresponding to this experiment are depicted in Supplementary Fig. S4.

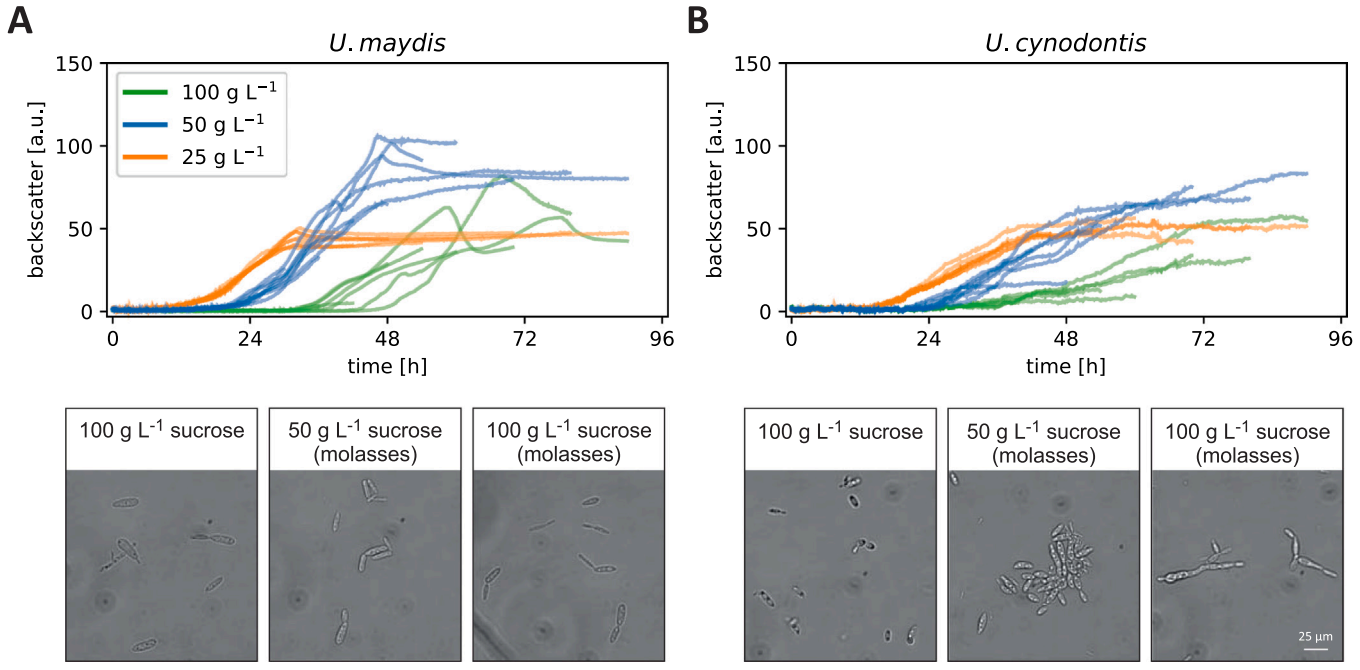


Fig. 5. Growth and microscopic images of A) *U. maydis* MB215 Δ*cyp3* ΔMEL ΔUA Δ*dgat* ΔP_{ria1}::P_{etef} Δ*fuz7* P_{etef}*mttA* and B) *U. cynodontis* Δ*fuz7*$_r$ Δ*cyp3*$_r$ P_{etef}*mttA* P_{ria1}*ria1* in MTM media containing different concentrations of beet molasses as the sole carbon and energy source. Microscale cultures were performed in a BioLector Pro at 30 °C, 1400 rpm, 85% humidity and 800 µL culture volume. The pH and DO profiles corresponding to this experiment are depicted in Supplementary Fig. S5.

range compared to those reported in previous studies, it can be assumed that not all nitrogen in molasses can be assimilated by *Ustilago sp.* because otherwise the induction of the itaconate synthesis would be suppressed by an oversupply of nitrogen [15,18,34,37]. The slower growth and lower yields obtained under the conditions with higher molasses concentrations are therefore probably due to impurities and other inhibiting components in the molasses. However, it cannot be ruled out that the itaconate production on 100 g L^{-1} sucrose equivalents was only delayed by the higher nitrogen concentration under this condition.

3.7. Growth and itaconate production of Ustilago sp. on supplemented molasses media

Since high molasses concentrations proved to have a negative effect on the growth of the fungus as well as the itaconate yield and lowering the molasses will also lower the overall titre, different molasses concentrations were supplemented with glucose to maintain a total sugar content of 100 g L^{-1} (Fig. 6). The concentrations of sucrose equivalents from molasses applied ranged from 25 to 75 g L^{-1}. For *U. cynodontis* the biomass produced under all three tested conditions fell short of the growth on commercial sucrose, while the values of *U. maydis* were comparable for the two experiments (cf. Table 1).

A corresponding inhibiting effect on the production of itaconate with *U. cynodontis* was visible, especially when high amounts of molasses were supplied (cf. Fig. 6). The sugars in the media, particularly the preferred carbon source glucose, were consumed significantly slower compared to the consumption of commercial sugars in the previous experiment (cf. Fig. 2). Only in the lowest molasses concentration and with the addition of 75 g L^{-1} glucose, was efficient production of itaconate possible, with a titre of 29.36 g L^{-1} and a yield of 0.39 g g^{-1} (cf.

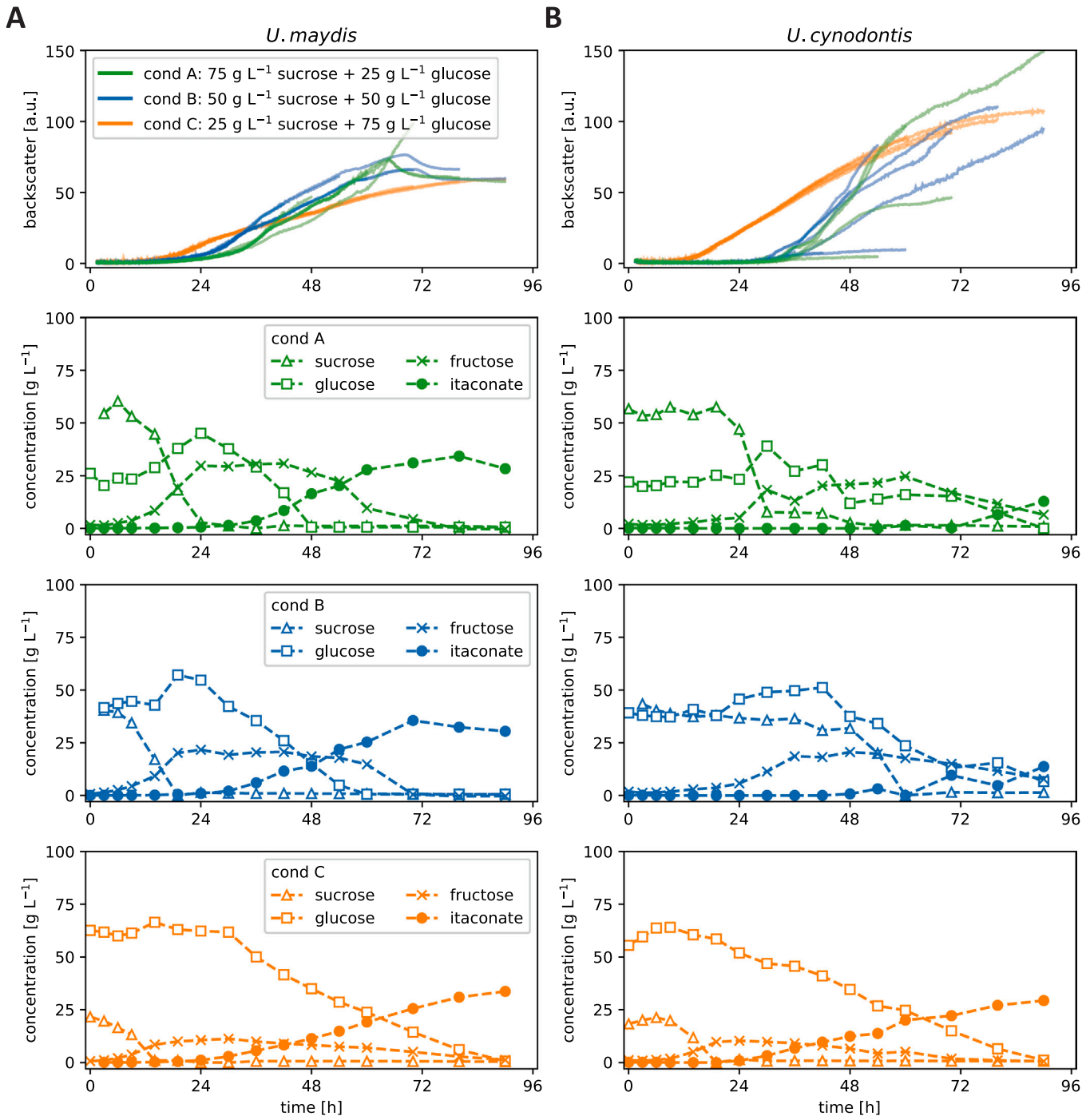


Fig. 6. Growth and itaconate production of A) *U. maydis* MB215 Δ*cyp3* ΔMEL ΔUA Δ*dgat* ΔP_{ria1}::P_{etef} Δ*fuz7* P_{etef}*mttA* and B) *U. cynodontis* Δ*fuz7*$_r$ Δ*cyp3*$_r$ P_{etef}*mttA* P_{ria1}*ria1* in MTM with different amounts of molasses supplemented with glucose to reach a final sugar concentration of 100 g L^{-1}. Microscale cultures were performed in a BioLector Pro at 30 °C, 1400 rpm and humidity controlled at 85%. The pH and DO profiles corresponding to this experiment are depicted in Supplementary Fig. S6.

Table 1). *U. maydis*, however, was able to produce approximately 30 g L^{-1} itaconate under all three conditions with yields exceeding those achieved under the same conditions on commercial sucrose and even on glucose.

3.8. Lab-scale fermentation of *U. maydis* on raw molasses

To demonstrate the scalability of the process for the production of itaconate using raw molasses, a lab-scale fed-batch fermentation was carried out with *U. maydis*. The cultivation was carried out in a 1 L bioreactor using the complex substrate as the sole carbon source for initial biomass growth as well as during the feed phase.

Within the first 23 h of the process, no increase in the oxygen transfer rate (OTR) or the carbon dioxide transfer rate (CTR) could be detected, indicating a prolonged lag phase (Fig. 7). After 23 h the conversion of sucrose to its monomers began, followed by an exponential increase in the respiration activity, which was accompanied by an increase in CDW from initially 2.4 g L^{-1} to 17.0 g L^{-1}. The growth phase culminated in a double peak of OTR and CTR with a maximum OTR of more than 63 mmol L^{-1} h^{-1} after 48 h. While the first peak likely represents the depletion of the assimilable nitrogen sources in the medium, the second peak indicates a lack of carbon sources in the medium. In agreement with the observed nitrogen limitation, itaconate accumulation started after 43 h.

The molasses feed was started when all sugars from the batch medium were consumed. This resulted in an immediate restoration of biomass growth as measured by increasing OTR, CTR and CDW. After stopping the feed, the respiration rates immediately dropped due to the lack of primary carbon sources. The respiratory data suggests that after the depletion of the sugars, lactate – which is contained in the molasses (cf. Fig. 1) – was consumed by the fungus. The exhaustion of the organic acid was observed after 95 h through a further drop in the respiration activity of *U. maydis*. Between the end of the feed and the depletion of lactate, the itaconate titre increased by 3.9 g L^{-1} to the final titre of 37.1 g L^{-1}. In the end, 54.2 g itaconate with a yield of 0.38 g g^{-1} and a space-time yield of 0.38 g L^{-1} h^{-1} were produced.

The results from the stirred tank reactors correspond largely to the findings from the microscale experiments. The itaconate yield reached with *U. maydis* on pure molasses was comparable to that achieved in the supplementation experiment, where molasses was spiked with commercial glucose to enhance the performance (cf. Fig. 6). Clearly, the process could be further optimised, for example by improving the timing at which the feed is started to avoid carbon limitation.

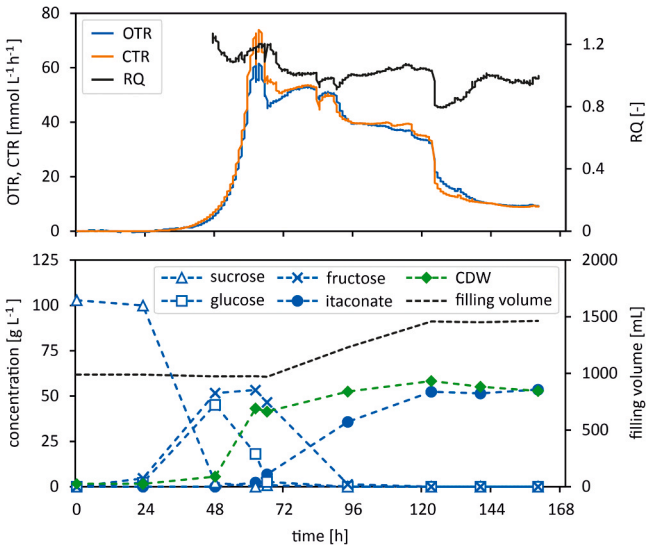


Fig. 7. Fed-batch fermentation of *U. maydis* MB215 Δ*cyp3* ΔMEL ΔUA Δ*dgat* ΔP_{ria1}::P_{etef} Δ*fuz7* P_{etef}*mttA* grown on molasses as the sole carbon source. The cultivation was performed in a 2 L Sartorius BIOSTAT® stirred tank reactor (Sartorius AG, Göttingen, Germany) with a filling volume of 1 L at 30 °C. Dissolved oxygen tension was kept at > 30% and the pH was kept constant at 6.5. RQ values are only shown for OTR values > 5 mmol L^{-1} h^{-1}. Samples were taken regularly and analysed via HPLC.

4. Conclusions

This study has shown that itaconate production with *Ustilago sp.* from molasses is feasible, especially using the engineered strain *U. maydis* MB215 Δcyp3 ΔP_{ria1}::P_{etef} Δfuz7 P_{etef}mttA, in particular because the strain – with its high invertase activity – has the ability to rapidly hydrolyse sucrose.

The strain at hand has an advantage over the animal pathogen *A. terreus,* since no pathogenicity towards animals or humans has ever been reported. However, with the highly engineered genome, it is classified as a GMO and thus can only be used in a contained space according to EU Directive 2009/41/EC [40]. Since the end product after downstream processing no longer contains cells, the distribution of biotechnologically produced itaconic acid is unproblematic.

Despite the high nitrogen load in molasses, *U. maydis* is able to efficiently produce itaconate, likely because not all nitrogen sources can be assimilated. Thus, the nitrogen in the molasses works to an advantage, since no additional nitrogen sources have to be added to the medium.

Although the titres reached on molasses in this study fall short of the values published on glucose [15,16], the yields reached with *U. maydis* under microscale and fed-batch fermentation conditions were comparable to those on commercial sugars. Further optimisation of the bioprocess parameters could achieve competitive itaconate titres from molasses. In conclusion, the use of molasses feedstock, when supplied from regionally planted and processed sugar beets, has the potential to significantly increase the sustainability of the itaconate production process.

Funding

This project has received funding from the Bio-based Industries Joint Undertaking (JU) under the European Union's Horizon 2020 research and innovation programme under grant agreement No 887711. The JU receives support from the European Union's Horizon 2020 research and innovation programme and the Bio-based Industries Consortium. Further funding was received by the German Federal Ministry of Education and Research (BMBF, project: "Modellregion, BioRevierPLUS: InBio, Innovationscluster Integrierte Bioraffinerie", grant no. 031B1135A).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2023.06.003.

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