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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

High oxygen tension increases itaconic acid accumulation, glucose consumption, and the expression and activity of alternative oxidase in *Aspergillus terreus*

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Abstract

Itaconic acid is a five-carbon dicarboxylic acid with an unsaturated alkene bond, frequently used as a building block for the industrial production of a variety of synthetic polymers. It is also one of the major products of fungal "overflow metabolism" which can be produced in submerged fermentations of the filamentous fungus *Aspergillus terreus*. At the present, molar yields of itaconate are lower than those obtained in citric acid production in *Aspergillus niger*. Here, we have studied the possibility that the yield may be limited by the oxygen supply during fermentation and hence tested the effect of the dissolved oxygen concentration on the itaconic acid formation rate and yield in lab-scale bioreactors. The data show that a dissolved oxygen concentration of 2% saturation was sufficient for maximal biomass formation. Raising it to 30% saturation had no effect on biomass formation or the growth rate, but the itaconate yield augmented substantially from 0.53 to 0.85 mol itaconate/mol glucose. Furthermore, the volumetric and specific rates of itaconic acid formation ameliorated by as much as 150% concurrent with faster glucose consumption, shortening the fermentation time by 48 h. Further increasing the dissolved oxygen concentration over 30% saturation had no effect. Moreover, we show that this increase in itaconic acid production coincides with an increase in alternative respiration, circumventing the formation of surplus ATP by the cytochrome electron transport chain, as well as with increased levels of alternative oxidase transcript. We conclude that high(er) itaconic acid accumulation requires a dissolved oxygen concentration that is much higher than that needed for maximal biomass formation, and postulate that the induction of alternative respiration allows the necessary NADH reoxidation ratio without surplus ATP production to increase the glucose consumption and the flux through overflow metabolism.

Keywords *Aspergillus terreus* · Itaconic acid · Alternative oxidase · Respiration · Submerged fermentation · Dissolved oxygen

Introduction

Itaconic acid (2-methylidenebutanedioic acid) is a renewable platform chemical for the industrial production of resins, acrylic polymers, super-absorbents, and anti-scaling agents (Bafana and Pandey 2018; Zhao et al. 2018; Cunha da Cruz et al. 2018; Kuenz and Krull 2018). The organic acid is formed by *Aspergillus terreus* during fermentation of fast metabolizable sugars (Kubicek et al. 2010). Although it can be generated at high yields merely subject to solubility restrictions (Karaffa et al. 2015), the costs of the biotechnological process remain too high to be an economically sustainable alternative for its traditional chemical synthesis from fossile resources (Bafana and Pandey 2018; Zhao et al. 2018). The performance of the biotechnological process may be improved

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by increasing the product yield from the fermentable carbon source, or by increasing the rate of acid accumulation during fermentation (i.e., effectively shortening the fermentation time).

Itaconic acid biosynthesis resembles that of citric acid in *Aspergillus niger* (Fig. 1a), the latter acid being a direct precursor of the former. This bioconversion involves three reactions: (a) dehydration of citrate to *cis*-aconitate, the intermediate of the aconitase activity (EC 4.2.1.3) normally converting citrate to isocitrate in the tricarboxylic acid cycle; (b) active transport of *cis*-aconitate out of the mitochondria catalyzed by a dedicated mitochondrial tricarboxylic acid exchanger; and (c) its conversion into itaconate by *cis*-aconitate decarboxylase (EC 4.1.1.6) in the cytosol (Li et al. 2011a; Steiger et al. 2013). One may therefore assume that the fermentative production of either acid would be enhanced under similar growth conditions, conductive to overflow metabolism in both *A. niger* and *A. terreus*.

Eventually, the acids produced are actively secreted into the culture medium. In most academic studies, low itaconic acid yields of 0.1–0.2 g per g glucose (0.15–0.22 mol/mol)

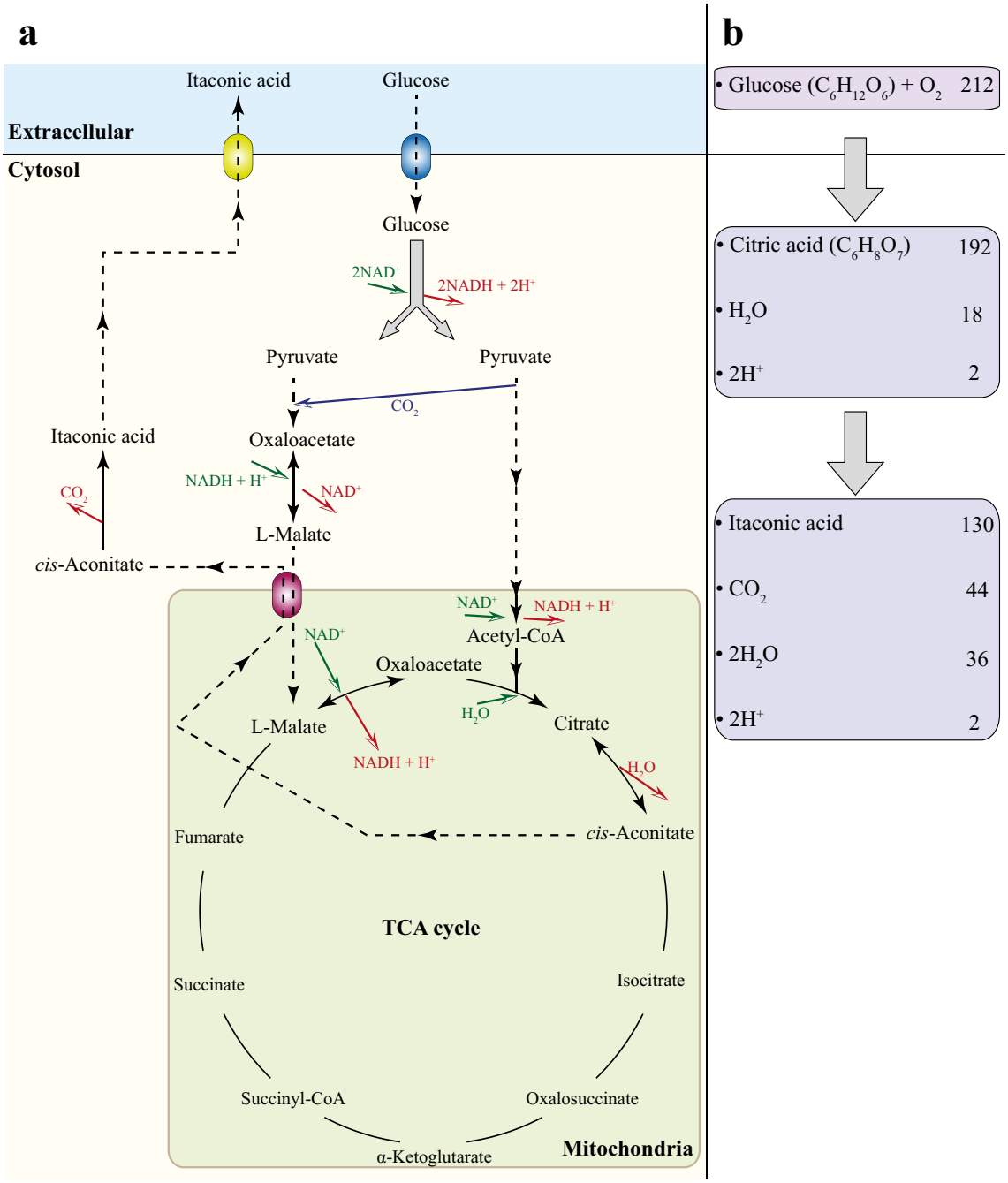

Fig. 1 Schematic representation of the biosynthesis of itaconic acid (**a**) and the overall mass balance (**b**) of the glucose-to-itaconate bioconversion in *A. terreus*

were obtained (Lai et al. 2007; Dwiarti et al. 2007; Saha et al. 2017; Wu et al. 2017), whereas citric acid yields in fermentations of *A. niger* are generally much higher and can reach 1 g per g glucose (0.9 mol/mol) (Karaffa and Kubicek 2003; Kubicek et al. 2010). These differences led some researchers to speculate that the genetic configuration of *A. terreus* may not be adequate to facilitate the production of higher titers of itaconic acid under fermentation conditions, and transplanted the clustered itaconic acid biosynthetic pathway genes into *A. niger*, a well-known industrial cell factory more amenable to molecular genetics (Steiger et al. 2013). However, recombinant *A. niger* strains expressing the pathway genes produced considerably less itaconic acid than *A. terreus* (van der Straat et al. 2014; Hossain et al. 2016; Yin et al. 2017). One possible explanation for the discrepancy between reported citric and itaconic acid yields is that the rigorous nutritional conditions known to be conductive to high-yield accumulation of citric acid in *A. niger* cultivations have not been applied in the study of itaconic acid synthesis in *A. terreus*. Two of such preconditions are a very high sugar concentration in the culture medium, and a paucity of manganese ions (Kubicek et al. 2010). The latter condition can only be attained by cation exchanger treatment of the carbon source and the other "non-metal" components of the medium, or by the supplementation of chemicals inhibiting or antagonizing uptake of manganese ions (Kubicek et al. 2010). We have recently shown that cultivation of *A. terreus* under these two nutritional conditions leads to accumulation of itaconic acid to similar high molar yields (up to 0.9 mol per mol; Karaffa et al. 2015), thus proving that the above preconditions are pivotal for efficient citric and itaconic acid biosynthesis in the respective *Aspergillus* species.

Another precondition for citric acid accumulation is the availability of dissolved oxygen at concentrations in clear excess of the (minimal) concentration needed for maximal biomass formation (Karaffa and Kubicek 2003; Kubicek et al. 2010). Citric acid (C6H8O7) contains one more oxygen atom than glucose (C6H12O6), which thus has to be recruited from another source. Citrate is a direct precursor of itaconate via *cis*-aconitate (Fig. 1a, b), and the two enzymatic steps after citric acid (NB. The dehydration reaction catalyzed by aconitase and *cis*-aconitate decarboxylation) are redox neutral. To date, the relevance of oxygen provision for itaconic acid accumulation in submerged fermentation has not been studied, but it has been reported that in capacitated *A. niger* strains, oxygen limitation would be beneficial rather than detrimental (Li et al. 2013).

In this work, we have investigated the influence of oxygen supply on itaconic acid biosynthesis in *A. terreus* in biofermentors. We demonstrated that increase of the dissolved oxygen concentration (DO) over that required for maximal biomass formation indeed increases the formation rate and molar yield of itaconic acid as well as the rate of glucose consumption.

Materials and methods

Fungal strain and cultivation conditions

Aspergillus terreus NRRL 1960 (CBS 116.46; ATCC 10020)—a recognized itaconic acid producing strain—was maintained on plates as described earlier (Kuenz et al. 2012). Apart from the dissolved oxygen (DO)—set at levels between zero and 40% of saturation—the submerged growth medium used was described in our previous work with *A. terreus* NRRL 1960 (Karaffa et al. 2015). D-Glucose was present at 12% (*w*/*v*) of initial concentration. To control the concentration of manganese(II) ions in the growth medium, 120 g of D-glucose was dissolved in distilled water and passed through a column (440 × 45 mm) of Dowex 50W-X8 (100/200-mesh) cation exchange resin. All other medium components were subsequently added to this glucose solution from sterile stock solutions made up with water treated with the cation exchanger. The final manganese(II) concentration in the medium was adjusted to 2 μg L^{-1} by the addition of appropriate volumes of a freshly prepared stock solution of $Mn(II)Cl_2$ tetrahydrate.

Bioreactor cultivations were carried out in 9-L glass fermentors (Inel, Budapest, Hungary) with a culture (working) volume of 6 L, equipped with two six-blade Rushton disc turbine impellers. All parts of the stirrer attachment consisted of high-quality steel that did not release manganese ions during sterilization and cultivation, but nevertheless we routinely verified this. Operating conditions were 33 °C and 0.75 vessel volume per minute (vvm) of aeration. The initial medium pH was adjusted to 3.0 with 3 M HCl before inoculation and was not controlled during fermentation. Dissolved oxygen (DO) levels were controlled by appropriately adjusting the impeller tip speed. DO, temperature, and impeller tip speed were controlled automatically by the regulatory units of the bioreactor. To minimize medium loss, the waste gas (from the headspace) was cooled in a reflux condenser connected to an external cooling bath (4 °C) before exiting the system. Cultures were inoculated with 5×10^6 *A. terreus* conidia per milliliter of medium from a freshly prepared, high-density spore suspension in a 1/10,000 Tween 20 solution. Pellet morphology at different DO levels was investigated as described previously (Karaffa et al. 2015).

All chemicals used were of analytical grade and purchased from Sigma-Aldrich (Budapest, Hungary), unless specified otherwise.

Analytical methods

Dry cell weight (DCW) was determined from 10-mL culture aliquots as described by Fekete et al. (2002). The biomass was harvested on a pre-weighted glass wool filter and washed with cold tap water, after which the filter was dried at 80 °C until constant weight. Dry weight data reported in the "Results" section are the means of two separate measurements, which never deviated by more than 14%. The concentration of D-glucose and itaconic acid in growth media was determined by high-pressure/performance liquid chromatography (HPLC; Gilson) with a proton exchange column (Bio-Rad Aminex HPX-87H$^+$) at 55 °C, using isocratic elution with 10 mM H_2SO_4 and refractive index (RI) detection. Manganese(II) ion concentrations were determined by using an inductively coupled plasma–quadrupole mass spectrometer (ICP-QMS; Thermo Fisher Scientific, Bremen, Germany), equipped with Hexapole Collision Cell Technology (CCT), as described by Karaffa et al. (2015). Biomass production rates (g of DCW h^{-1}) were calculated from the increase in DCW over the time elapsed between two subsequent samplings (i.e., sampling time points); the highest of the thus obtained values was taken as the maximal specific growth rate of the culture. Likewise, glucose utilization rates (g g_{DCW}^{-1} h^{-1}) were calculated from the biggest decrease in residual concentrations between two subsequent samplings. The measurement of the mycelial respiration rates, including that of alternative respiration, was performed with an oxygraphic electrode (Strathkelvin Instruments Ltd., North Lanarkshire, UK) at 37 °C, according to the manufacturer's instructions (Molnár et al. 2018). Potassium cyanide (1 mM) was used to selectively inhibit the cytochrome C oxidase. After oxygen consumption measurements, the biomass utilized in these assays was harvested and DCW was determined, allowing calculation of the specific oxygen uptake rates.

Reproducibility

All the analytical data presented are the means of three to five independent experiments (fermentations). Data were analyzed and visualized with SigmaPlot (Systat Software, San Jose, CA, USA), and for each procedure, standard deviations (SDs) were determined.

Transcript analysis

Total RNA isolation and subsequent Northern analysis were essentially conducted as described previously for *A. nidulans* (Fekete et al. 2016). The oligonucleotide primers used to PCR digoxigenin-labeled probes for the transcripts of the two putative alternative oxidase genes in the *A. terreus* genome (see "Results" section) are given in Supplementary Table S1; genomic DNA was used as the template.

Results

Increased dissolved oxygen increases itaconic acid accumulation and glucose consumption but not biomass formation

To test our hypothesis that oxygen is directly implicated in itaconic acid biosynthesis, we cultivated *A. terreus* under conditions optimal for citric acid accumulation in *A. niger* with 12% (*w*/*v*) glucose as a carbon source, at nine different concentrations of dissolved oxygen (ranging from zero—oxygen limitation—to 40% of saturation). As can be seen from Table 1, a DO concentration of 2% saturation was already sufficient for maximal biomass formation, and a further DO increase neither augmented nor lowered it. Biomass formation remained constant at a specific growth rate of 0.02 h^{-1}, reaching a final biomass concentration of 17–18 g DCW L^{-1}. Oxygen supply in the fermentors was regulated by the stirrer speed, and the increased shear force at higher DO concentrations may have consequences for pellet morphology which in turn could affect the oxygen mass transfer into the bioreactor. We microscopically inspected the growing biomass at different timepoints, but found essentially no morphological differences between the pellets formed at different stirring rates (some examples provided in Supplementary Fig. S2).

Further increase in the concentration of DO over 2% saturation, however, resulted in an increase in the final concentration of itaconic acid by 38%, from 45.9 g L^{-1} up to 73.6 g L^{-1}, which is equivalent to an increase of the molar yield ($Y_{P/S}$) from 0.53 up to 0.85. The increase in $Y_{P/S}$ coincided with ever higher rates of the glucose consumption by the biomass (Table 1). This implies that under these conditions (DO > 2% saturation), the extra carbon source consumed over the amount necessary to reach the maximum growth rate is channeled directly into overflow metabolism: The more oxygen supplied, the more glucose converted into itaconic acid. The maximal conversion efficiency was obtained at a DO concentration of 30% saturation, and a further increase in the DO concentration (to 40%) did not further raise the molar yield of accumulated itaconic acid (Table 1).

Increased dissolved oxygen increases the rate of itaconic acid accumulation and reduces the fermentation time

The increase in $Y_{P/S}$ of itaconic acid coincided with an increase in the rate of glucose uptake, again until 30% DO (Fig. 2). This accelerated glucose uptake suggested that the rate of itaconic acid accumulation would also augment since no extra biomass is generated above 2% saturation. To test

Table 1 Biomass (DCW) and itaconic acid (IA) production as well as derived kinetic parameters of *Aspergillus terreus* NRRL 1960 cultivations as a function of the dissolved oxygen (DO) level. Mycelia grew under submerged conditions in an optimized IA-producing medium initially containing 120 g L^{-1} D-glucose as the sole carbon source (see the "Materials and methods" section). The fermentations at 2 and 30% DO saturation (boxed datasets) were subjected to further analysis (see Fig. 3)

Dissolved oxygen (% of saturation)	Maximal DCW (g L^{-1})	DCW yield ($Y_{x/s}$)	Final IA concentration (g L^{-1})	Molar IA yield ($Y_{p/s}$)	Glucose utilization rate (g L^{-1} h^{-1})	Growth rate (g_{DCW} h^{-1})
100 rpm[a]	16.9 ± 0.5	0.14 ± 0.004	42.5 ± 1.7	0.49 ± 0.02	0.48 ± 0.03	0.02 ± 0.004
200 rpm[a]	17.4 ± 0.7	0.14 ± 0.006	45.1 ± 2.6	0.52 ± 0.03	0.49 ± 0.04	0.02 ± 0.003
2	16.5 ± 0.6	0.14 ± 0.005	45.9 ± 2.6	0.53 ± 0.03	0.50 ± 0.04	0.02 ± 0.003
5	16.9 ± 0.8	0.14 ± 0.007	46.8 ± 3.5	0.54 ± 0.04	0.53 ± 0.04	0.02 ± 0.003
10	17.1 ± 0.5	0.14 ± 0.004	51.1 ± 4.4	0.59 ± 0.05	0.57 ± 0.04	0.02 ± 0.002
15	17.9 ± 0.8	0.15 ± 0.007	57.2 ± 4.4	0.66 ± 0.05	0.61 ± 0.03	0.02 ± 0.002
20	16.8 ± 0.4	0.14 ± 0.003	65.0 ± 4.4	0.75 ± 0.05	0.66 ± 0.04	0.02 ± 0.002
30	17.2 ± 0.5	0.14 ± 0.004	73.6 ± 4.4	0.85 ± 0.05	0.71 ± 0.05	0.02 ± 0.002
40	17.6 ± 0.7	0.15 ± 0.006	73.6 ± 4.4	0.85 ± 0.05	0.71 ± 0.04	0.02 ± 0.002

[a] Fixed agitation rate throughout the cultivation. During these fermentations, DO levels continuously changed and dropped to zero % of saturation for different time lapses during the growth stage (see Fig. S1). For further details, see the "Results" section

this, two fermentations were conducted under identical conditions at different DO concentrations (2 and 30% saturation, respectively), and biomass and itaconic acid formation, glucose consumption, and DO were monitored with time. As can be seen from Fig. 3, the maximal rate of itaconic acid accumulation at a DO concentration of 2% was 0.2 g L^{-1} h^{-1}, whereas it reached 0.51 g L^{-1} h^{-1} at 30% saturation. Consequently, the fermentation time lapse to achieve the maximal concentration of itaconic acid was considerably shortened (216 h vs 264 h, at 30% and 2% saturation, respectively). We therefore conclude that the DO in the culture not only influences the molar yield of the glucose to itaconic acid bioconversion but also affects the formation rate.

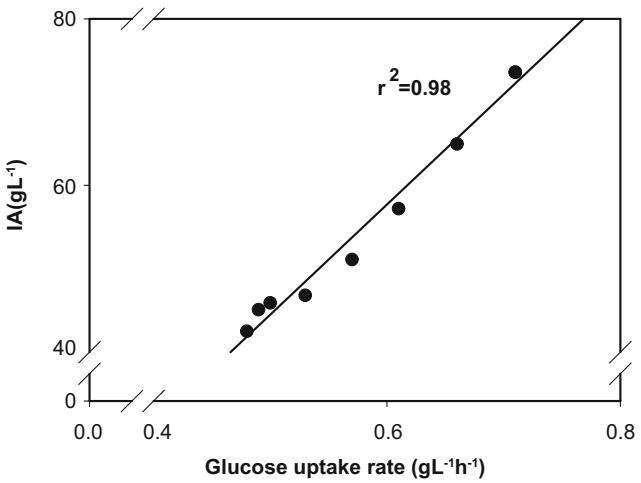

Fig. 2 The relationship between glucose uptake rate (g L^{-1} h^{-1}) and final itaconic acid (IA) concentration (g L^{-1}) in *A. terreus* fermentations with increasing DO concentration. The plot is deduced from the data given in Table 1. The coefficient r^2 of the fitted regression line indicated a one-on-one correlation between the two parameters

Increased dissolved oxygen stimulates the operation of an alternative respiratory pathway

The results described above—i.e. that oxygen availability influences the rate of itaconic acid biosynthesis and glucose uptake once beyond the DO concentration needed to reach the maximum growth rate—imply that the fungal cell must have an efficient mechanism to reoxidize NADH formed during glycolysis and mitochondrial pyruvate oxidation, and recycle catabolic reducing equivalents without the production of surplus ATP. During citric acid fermentation in *A. niger*, the operation of an alternative mitochondrial terminal oxidase uncouples NADH oxidation from ATP formation via oxidative phosporylation by bypassing the proton-pumping cytochrome complexes III and IV of the electron transfer chain (for a review, Young et al. 2013). Unlike the cytochrome C oxidase complex, alternative oxidase (AOX; EC 1.10.3.11) is insensitive to cyanide inhibition. We therefore tested whether alternative respiration also occurs in *A. terreus* under conditions conductive to itaconic acid synthesis by measuring the ratio of cyanide-resistent respiration and total respiration (Table 2). In mycelia growing at a DO of 2% saturation, the alternative respiratory pathway accounted for 15% of the total respiration. In contrast, raising the DO concentration led to a gradually increasing contribution of the cyanide-resistant fraction, culminating in a threefold increase in alternative oxidase activity that accounted for 47.7% of the total respiration at a DO of 30% saturation. Note that the total respiration remained essentially at the same level (28.1–29.2 μM min^{-1} $gDCW^{-1}$: the variation remains within the experimental standard deviation) in the seven DO controlled fermentations; hence, the biomass did not

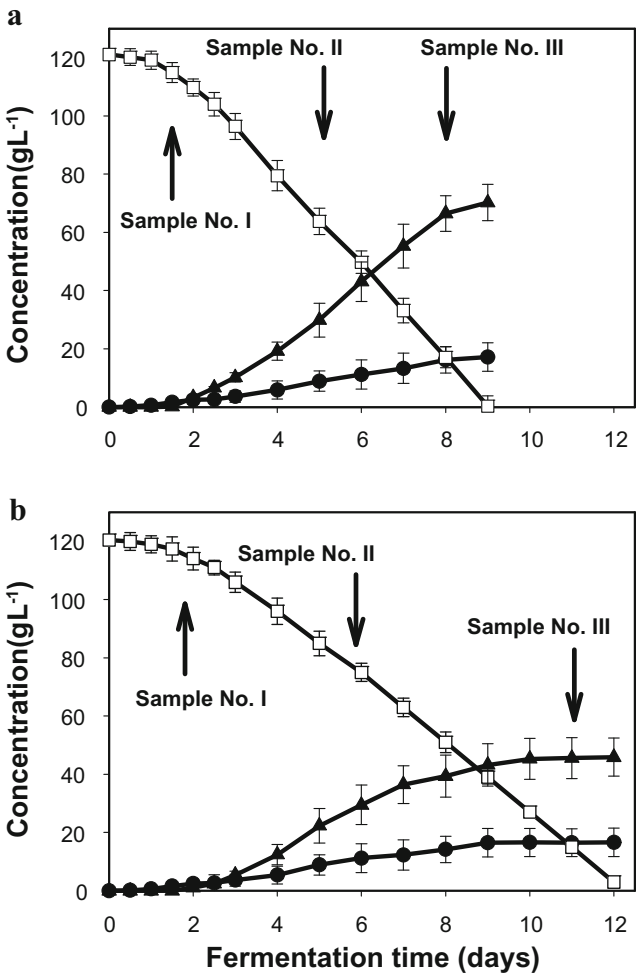

Fig. 3 Kinetics of biomass (●), itaconic acid (▲), and residual D-glucose (□) concentrations in the medium in the selected submerged fermentations of *A. terreus* at 30% (**a**) and 2% (**b**) controlled DO saturation. Mycelia grew under submerged conditions in an optimized IA-producing medium initially containing 120 g L^{-1} D-glucose as the sole carbon source. For full details, see the "Materials and methods" section. The three variables share the same y-axis as they are expressed in the same unit (g L^{-1}). Biomass was sampled for transcript analysis (Fig. 4) at the indicated time points for each of the two fermentations. For further details, see the Legend to Fig. 4

respire more or faster (i.e., did not use more molecular oxygen) with increasing itaconic acid formation rates and $Y_{P/S}$.

Expression of the *aoxA* gene correlates with dissolved oxygen tension and itaconic acid formation

A gene encoding an alternative oxidase (AOX) has been demonstrated to be expressed in *A. niger* during citric acid production (Zehentgruber et al. 1980; Hattori et al. 2009). We verified whether this would also be the case in *A. terreus* itaconic acid fermentation. To this end, we screened the genome sequences of *A. terreus* strain NIH2624 (GenBank Whole Genome Shotgun sequencing project Master Accession AAJN01000000), and identified two putative AOX encoding genes, abbreviated as *aoxA* and *aoxB*, at auto-annotated loci ATEG_05999 and ATEG_07440, respectively (data not shown—reported briefly in de Vries et al. 2017). We tested the expression of both during itaconic acid accumulation at high (30%) and low (2%) DO concentration, and found that the transcript levels of *aoxA* are considerably higher at 30% saturation (Fig. 4). *aoxB* expression, on the other hand, could not be observed under these conditions. These results strongly suggest that the *aoxA* gene (locus ATEG_05999) is responsible for the alternative respiration observed under all the tested conditions.

Discussion

Molecular oxygen is a key substrate for growth and metabolism of aerobic microoorganisms, and indispensable for the industrial production of metabolites or enzymes. The impact of oxygen supply has been demonstrated for several fermentative cultivations (Manfredini et al. 1983). However, in most of these studies the necessity of oxygen supply has been considered only with respect to generating the energy required to fuel anabolic metabolism, i.e., biomass formation and biosynthesis of metabolites (Garcia-Ochoa and Gomez 2009). In itaconic and citric acid formation, the action of pyruvate carboxylase and the excretion of the product of overflow metabolism into the medium require extra ATP. The possibility that molecular oxygen may serve as a co-substrate in the formation of microbial metabolites beneficial to mankind that have a higher O/H ratio than the fermentation substrate has not received much attention to date. A notable example is gluconic acid production in *A. niger*, as glucose oxidase is a flavoprotein that uses oxygen as a co-substrate to recycle the FAD redox co-factor and produces peroxide as a noxious (by)product (Traeger et al. 1991). The synthesis of the mycotoxins sterigmatocystin and aflatoxin involves the action of seven mono- and dioxygenases (EC 1.13/EC 1.14) (Yabe and Nakajima 2004), oxidoreductases that transfer (one or both) oxygen atom(s) from molecular oxygen to the main substrate.

In *A. niger* citric acid fermentation, the continuous provision and availability of high concentrations of DO in the culture is one of the established preconditions that drive overflow metabolism and boost product accumulation (Karaffa and Kubicek 2003). As citric acid is a direct precursor of itaconic acid, we have investigated the involvement of oxygen in the production of itaconic acid in *A. terreus*. Using different concentrations of DO between 2 and 30% of saturation, we found that at 2% saturation DO is already sufficiently available for maximal biomass formation, and a further increase has neither positive nor negative effects on the rate of growth. The growth-independent augmentation of both the itaconic acid yield and the rate of its accumulation at DO concentrations beyond the rather low 2% of saturation indicates that oxygen

Table 2 Total and cyanide-resistant specific respiration rates, and the ratio of the cyanide-resistant fraction of *Aspergillus terreus* NRRL 1960 cultures, at nine different dissolved oxygen (DO) concentrations. Samples for assays were taken four days after inoculation, at the rapid growth stage of submerged fermentations in the optimized itaconic acid (IA)-producing medium initially containing 120 g L^{-1} D-glucose as the sole carbon source. The fermentations at 2 and 30% DO saturation (boxed datasets) were subjected to further analysis (see Fig. 3)

Dissolved oxygen (% of saturation)	Total respiration ($\mu M\ min^{-1}\ g_{DCW}{}^{-1}$)	Cyanide-resistant respiration ($\mu M\ min^{-1}\ g_{DCW}{}^{-1}$)	Cyanide-resistant fraction (%)
100 rpm[a]	19.4 ± 1.1	3.0 ± 0.2	15.4
200 rpm[a]	23.4 ± 1.0	3.6 ± 0.3	15.4
2	28.4 ± 1.2	4.5 ± 0.2	15.8
5	28.9 ± 1.1	6.3 ± 0.5	21.8
10	29.1 ± 1.5	8.1 ± 0.6	27.8
15	28.8 ± 1.6	11.5 ± 0.6	39.9
20	28.1 ± 1.8	12.4 ± 0.8	44.1
30	29.2 ± 1.3	13.8 ± 0.7	47.3
40	28.7 ± 1.1	13.7 ± 0.8	47.7

[a] Fixed agitation rate throughout the cultivation. During these fermentations, DO levels continuously changed and dropped to zero % of saturation for different time lapses during the growth stage (see Fig. S1). For further details, see the "Results" section

is essentially involved in the metabolic overflow of itaconic acid in *A. terreus* cultures. The parallel acceleration of glucose consumption is fully consistent with this conclusion. These findings strongly suggest that carbon flux and oxygen supply must be in sync to enable high itaconic acid yields and production rates in bioreactor fermentations. Nevertheless, increasing the DO over 30% saturation had no further effect on itaconic acid formation nor on glucose consumption, indicating that one of the enzymes or transport systems involved in overflow metabolism of itaconic acid becomes limiting. It would be interesting to test whether a further increase in the medium concentration of glucose would result in a further DO-dependent increase in itaconic acid yield and rate of synthesis. This was however out of the scope of this paper.

In this work, we also show that raising the DO concentration in the growth medium resulted in increased activity of the alternative respiratory pathway in *A. terreus*, raising the contribution of alternative respiration to the total respiration. This pathway transfers electrons from NADH via reduced ubiquinone directly to oxygen, without pumping protons out of the mitochondrial matrix, and thereby bypasses the standard electron transfer chain and subsequent ATP formation driven by the proton gradient over the inner mitochondrial membrane (Young et al. 2013). Alternative respiration is induced under conditions in which the cytochrome-mediated respiration is impaired or absent, e.g., in the filamentous fungi *Aspergillus fumigatus* and *Podospora anserina* (Grahl et al. 2012; Bovier et al. 2014), but in addition appears to serve other purposes, such as in temperature-, homeostasis-, and stress responses across different kingdoms (animals, plants, fungi) (Selinski et al. 2018; McDonald and Gospodaryov 2018; Saha et al. 2016; Li et al. 2011b). A key protein in the alternative respiratory pathway is alternative oxidase (AOX), which in fungi has been shown to be responsive to oxidative and osmotic stress (Grahl et al. 2012; Cárdenas-Monroy et al. 2017; Garcia-Neto et al. 2017; Honda et al. 2012). Our data show that the ubiquitous cytochrome-mediated

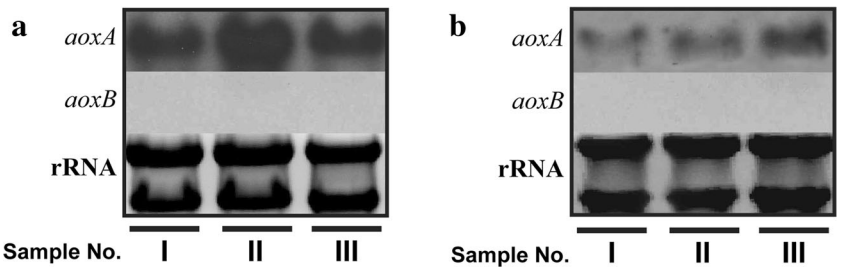

Fig. 4 Transcript analysis of the *aoxA* and *aoxB* genes at DO concentrations of 30% (**a**) and 2% saturation (**b**), respectively. Samples were taken early in growth (sample no. I), during the fast growth phase (sample no. II) and late in the fermentation (sample no. III): [at 30% saturation], 2, 5, and 8 days after inoculation; [at 2% saturation], 2, 6, and 11 days. The times of biomass sampling are also indicated in Fig. 3. Both panels come from the same Northern analysis (same membrane, hybridization, and exposure time). The figure shows representative results from three independent experiments (biological triplication)

electron transport chain remains active during itaconic acid fermentation—at 30% DO saturation, it still accounts for more than half of the total respiration. However, transfer of filamentous fungi to a stirred and aerated fermentor provokes oxidative stress responses (Bai et al. 2003). This induction may be enhanced by the paucity of manganese in the fermentation medium; the mitochondrial Mn(II)-dependent superoxide dismutase (EC 1.15.1.1) is a key antioxidant enzyme actively converting superoxide radicals into less harmful oxygen species (Higgins et al. 2002). We propose that the positive correlation between DO concentration and *aoxA* gene expression could constitute an intrinsic oxidative stress response. Nevertheless, it was recently shown that (artificial) inhibition of oxidative phosporylation by antimycin A (NB. An inhibitor of cytochrome C dehydrogenase—complex III of the electron transport chain) enhances the yield in citric acid fermentation in *A. niger* (Wang et al. 2015). In the contrary, specific inhibition of AOX activity by salicylhydroxamate decreases the rate of citric acid accumulation (Kubicek et al. 1980; Kirimura et al. 2000). To our knowledge, there is no conclusive evidence published to suggest that the positive correlation between operation of the alternative respiratory pathway and organic acid production by *Aspergillus* spp. is functional or fortuitous. An AOX encoding gene from *A. niger* has been cloned (Kirimura et al. 1999), but its deletion and overexpression have not been reported. On the other hand, it has been reported that *Acremonium chrysogenum* alternative oxidase has a rather low affinity for molecular oxygen (Kozma and Karaffa 1996). Increasing the DO concentration under conditions conductive to itaconate accumulation in *A. terreus* could thus result in more efficient oxygen reduction by alternative oxidase, increasing the flux through alternative respiration and consequently, lowering the synthesis of (surpus) ATP during overflow metabolism.

In summary, we have shown that a DO concentration in the nutrient medium which surpasses that required for maximal biomass formation is conductive to high yields of itaconic acid and higher specific formation rates in *A. terreus* fermentations. As a consequent improvement of the production process, the time lapse to achieve the maximal concentration of itaconic acid—equivalent to a molar yield of 0.85—on a well-defined production medium was shortened by 2 days (equivalent to 18%) when conducting the fermentation at a DO concentration of 30% saturation. Our current results, and those from earlier work (Karaffa et al. 2015), demonstrate that four of the nutritional preconditions known to promote production of citric acid in *A. niger* at high yields (i.e., high sugar concentrations, low medium pH, manganese(II) paucity, and high DO concentrations) also expedite high-titer itaconic acid production in *A. terreus*. This is in accordance with the early patents by Miles Corporation (Batti and Schweiger 1963; Batti 1967) in which essentially the same medium and growth conditions were used to produce these organic acids in cultivations of the respective species of *Aspergillus*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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

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