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# A deficiency of manganese ions in the presence of high sugar concentrations is the critical parameter for achieving high yields of itaconic acid by *Aspergillus terreus*

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**Abstract** Itaconic acid (IA), an unsaturated dicarboxylic acid with a high potential as a platform for chemicals derived from sugars, is industrially produced by large-scale submerged fermentation by *Aspergillus terreus*. Although the biochemical pathway and the physiology leading to IA is almost the same as that leading to citric acid production in *Aspergillus niger*, published data for the volumetric (g $L^{-1}$) and the specific yield (mol/mol carbon source) of IA are significantly lower than for citric acid. Citric acid is known to accumulate to high levels only when a number of nutritional parameters are carefully adjusted, of which the concentration of the carbon source and that of manganese ions in the medium are particularly important. We have therefore investigated whether a variation in these two parameters may enhance IA production and yield by *A. terreus*. We show that manganese ion concentrations< 3 ppb are necessary to obtain highest yields. Highest yields were also dependent on the concentration of the carbon source (D-glucose), and highest yields (0.9) were only obtained at concentrations of 12–20 % (*w*/*v*), thus allowing the accumulation of up to 130 g $L^{-1}$ IA. These findings perfectly mirror those obtained when these parameters are varied in citric acid production by *A. niger*, thus showing that the physiology of both processes is widely identical. Consequently, applying the fermentation technology established for citric acid production by *A. niger* citric acid production to *A. terreus* should lead to high yields of IA, too.

**Keywords** *Aspergillus terreus* · Itaconic acid · Fermentation · Manganese ions · D-Glucose · Specific yield · Volumetric yield

## Introduction

Itaconic acid (IA), originally discovered in 1837 as a thermal decomposition product of citric acid (Baup 1837), is a molecule with two carboxyl groups, a conjugated double bond and an activated carboxyl group by an ethyl–methyl group. These features make it an interesting building block for the synthesis of various chemicals with a broad range of applications and particularly in manufacturing plastics or resins (Okabe et al. 2009). Industrial production of IA occurs entirely by submerged fermentation with the fungus *Aspergillus terreus* (Batti and Schweiger 1963; Nubel and Ratajak 1962; von Fries 1966) and is one of the major examples of organic acid production by *Aspergillus* spp. due to metabolic overflow of primary metabolism.

The best known organic acid produced by *Aspergillus* is citric acid, which reaches final concentrations of up to 200 g $L^{-1}$, representing a molar yield ($Y_{p/s}$) of 0.9 (Karaffa and Kubicek 2003; Kubicek et al. 2010). These product concentrations require not only high-yielding mutant strains but also a delicate balance of nutrients in the medium that cause the deregulation of metabolism necessary for citric acid overflow (Karaffa and Kubicek 2003; Kubicek et al. 2010).

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Among those, variations in the concentration of manganese ions have shown to produce the strongest effects and to be interrelated with sugar concentration, i.e. increasing the carbon source from 5 to 15 % (*w*/*v*) significantly increased the molar yield, but only if the concentration of manganese ions was below a certain threshold (Shu and Johnson 1948; Clark et al. 1966). Notably, the critical concentration of manganese ions is in a range that can easily be exceeded by nutrients of even analytical grade, and thus they have to be actively removed or antagonized (cf. Röhr et al. 1996).

IA biosynthesis occurs along the same metabolic pathways as citric acid (Steiger et al. 2013) yet involves one additional enzyme, *cis*-aconitate decarboxylase encoded by the *cadA* gene, which is flanked by genes encoding a putative mitochondrial transporter (*mttA*) and a putative plasma membrane transporter (*mfsA*), both of which may be involved in mitochondrial *cis*-aconitate and plasma membrane itaconic acid transport (Li et al. 2013; Fig. 1). Titers and molar yields of itaconic acid reported for *A. terreus* in the literature yet are in most cases significantly lower than those for citric acid produced by *A. niger* (reviewed by Okabe et al. (2009)). Consequently, there have been several attempts to use gene transfer to convert *A. niger* into an IA producer (Blumhoff et al. 2013; Li et al. 2013; Van der Straat et al. 2014). Up to now, however, IA titers reported by these authors were also very low, even lower than with *A. terreus*.

A detailed screening of the recent literature revealed that, despite several attempts to increase IA production by *A. terreus* by optimizing the medium and fermentation parameters (Kuenz et al. 2012; Hevekerl et al. 2014), the effect of the concentration of manganese ions was never critically investigated. The objective of this paper therefore was to test whether IA can be produced by *A. terreus* in yields and final concentrations similar to citric acid, if the manganese ion and sugar concentrations are carefully controlled.

## Materials and methods

### Fungal strain and cultivation conditions

*A. terreus* NRRL 1960 (CBS 116.46, ATCC 10020), a standard high-producer strain, was kindly provided by Prof. Peter J. Punt (Microbiology & Systems Biology, TNO, Zeist, The Netherlands). The conditions for strain maintenance have been described earlier (Kuenz et al. 2012). Per litre of distilled water, the medium contained 0.1 g $KH_2PO_4$, 3 g $NH_4NO_3$, 1 g $MgSO_4 \times 7H_2O$, 5 g $CaCl_2 \times 2H_2O$, 1.67 mg $FeCl_3 \times 6H_2O$, 8 mg $ZnSO_4 \times 7H_2O$, and 15 mg $CuSO4 \times 7H_2O$. The carbon source (i.e. D-glucose) was used at concentrations from 1 to 20 % (*w*/*v*). Glucose and all other supplements were added from sterile stock solutions.

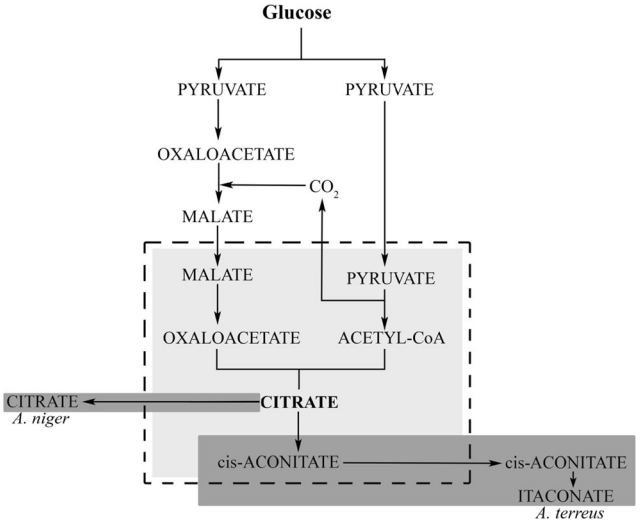


**Fig. 1** Metabolic scheme and compartmentalization of key steps in the conversion of D-glucose into citric acid and itaconic acid (by *A. niger* and *A. terreus*, respectively, based on information from Kubicek (1988) and Jaklitsch et al. (1991)). The mitochondrial steps are surrounded by a *dotted line* and highlighted by a *light grey* background. Reactions specific for *A. niger* or *A. terreus* are highlighted by a *dark grey* background and indicated by the species name

To control the concentration of manganese ions in the growth medium, depending on the condition, 10–200 g of D-glucose was dissolved in 1 L distilled water and passed through a column (440×45 mm) of Dowex 50 W-X8 (100/200-mesh) cation exchange resin. All other nutrient components were subsequently dissolved in this glucose solution. The final manganese ion concentration in the medium was adjusted by the addition of appropriate volumes of a stock solution of $MnCl_2 \times 4H_2O$.

Shake flask cultivations were performed with 100 mL medium in 500-mL Erlenmeyer flasks at 33 °C in a rotary shaker operating at 200 rpm. 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 cultivation. Operating conditions were 33 °C and 0.75 vvm aeration. The initial pH value was adjusted to 3.0 before inoculation and was not further controlled. Dissolved oxygen (DO) levels were maintained at 30 % saturation by appropriately adjusting the impeller tip speed. DO, temperature and impeller tip speed were controlled 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 $5 \times 10^6$ *A. terreus* conidia per mL of medium.

All chemicals used, unless indicated otherwise, were of analytical grade and purchased from Sigma-Aldrich.

### Analytical methods

Mycelial dry weight (DCW) was determined from 2- to 25-mL culture aliquots in shake-flask and bioreactor cultivations, respectively. The biomass was harvested and washed on a preweighted glass wool filter by suction filtration and filter-dried at 80 °C until constant weight. Dry weight data reported in the "Results" section are the average of the two separate measurements, which never deviated more than 14 %.

The concentration of D-glucose and IA 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 detection. The concentrations given are the average of two independent measurements, which never deviated more than 3 %.

For the determination of manganese ion concentrations in the growth media, inductively coupled plasma quadruple mass spectrometer (ICP-QMS; Thermo Fisher Scientific, Bremen, Germany), equipped with Hexapole Collision Cell Technology (CCT), was used. A gas mixture of 7 % hydrogen and 93 % helium was applied as collision/reaction gas at a flow rate of 6 mL min$^{-1}$. The instrument was controlled by a PlasmaLab software (ver. 2.5.10.319, Thermo Fisher Scientific). Calibration curves were set up by appropriate dilutions of a mono-elemental manganese reference solution (1000 mg L$^{-1}$ Mn(II), Scharlab S. L., Spain). Recovery was in the range of 95–100 %. The samples were analysed at *m*/*z* 55 for Mn(II) and *m*/*z* 103 for the internal standard element rhodium, which had a concentration of 20 μg L$^{-1}$ in all solutions. Typical instrument settings are summarized in Table S1.

Fungal morphology was investigated by means of an AxioVision AC quantitative image analyser system. To increase contrast and visibility, lactophenol cotton blue (Fluca) was added to the samples in a final concentration of 10 %. Stained samples were analysed under a Zeiss AxioImager microscope, equipped with AxioCam MRc5 camera.

### Reproducibility

All the analytical data presented are the means of three to seven independent experiments (fermentations). Data were analysed and visualized with SigmaPlot software (Jandel Scientific), and for each procedure, standard deviations (SDs) were determined.

## Results

### Manganese deficiency has multiple effects on A. terreus physiology

As a prerequisite to this study, we first quantified the concentration of manganese ions in the culture medium with and without cation exchange treatment of the sugar. Kisser et al. (1980) reported that a concentration of manganese ions higher than 5.6 μg L$^{-1}$ already decreases the final yield of citric acid in *A. niger*. Table 1 shows that, without cation exchange treatment, only distilled or tap water was clearly below this level. However, even only a 1 % (*w*/*v*) solution of D-glucose contained the double concentration of this treshhold, and 15 % (*w*/*v*) D-glucose exceeded this value 30-fold. The cation exchange-treated glucose solutions, however, exhibited manganese concentrations clearly below the critical 5.6 μg L$^{-1}$.

Having established nutrient conditions with defined manganese ion concentrations, we now grew *A. terreus* on 1 % (*w*/*v*) glucose in the absence of any further manganese additions, and in the presence of 0.25 mg L$^{-1}$ manganese ions, which is a concentration usually used for fungal submerged cultivations (Dahod 1999). As can be seen in Fig. 2a, b, *A. terreus* grew faster in the presence of manganese ions, finally reaching a biomass concentration of 5.1±0.4 g L$^{-1}$ that roughly corresponded to a biomass yield ($Y_{x/s}$) of 0.50 (Fig. 2b). In contrast, manganese ion-deficient fungal cultures exhibited a maximum biomass concentration of 3.6±0.3 g L$^{-1}$ that corresponded to a $Y_{x/s}$ of 0.35 (Fig. 2a). Similarly, D-glucose uptake was more rapid in the manganese ions-sufficient cultures, and consequently, carbon source exhaustion occurred approximately 24 h earlier than in the manganese ion-deficient cultures.

The presence of manganese ions completely prevented the accumulation of IA, whereas under manganese ion limitation, a small concentration (0.86 g L$^{-1}$) was formed that corresponded to a specific molar yield (moles IA/moles D-glucose consumed) of 0.12.

### Specific IA yield increases at higher D-glucose concentrations

The previous experiment demonstrated that IA is formed only under manganese ion-deficient conditions, but the specific yield ($Y_{p/s}$) of IA on 1 % (*w*/*v*) D-glucose was only 0.12. In citric acid production by *A. niger*, the $Y_{p/s}$ drastically increases when the sugar concentration is raised>5 %

**Table 1** Manganese ion concentration (in μg L$^{-1}$) in distilled water, tap water, and D-glucose solutions with and without cation exchange treatment.

| | Tap water | Destilled water | 1 % D-glucose | 15 % D-glucose |
|---|---|---|---|---|
| Treated[a] | 0.46±0.03 | <0.05 | 1.78±0.05 | 2.54±0.15 |
| Untreated | 3.25±0.05 | 0.43±0.02 | 10.12±0.25 | 149.5±2.12 |

[a] Treated samples were purified over a Dowex cation exchange resin, thereby removing manganese ions (see "Materials and methods" for details)

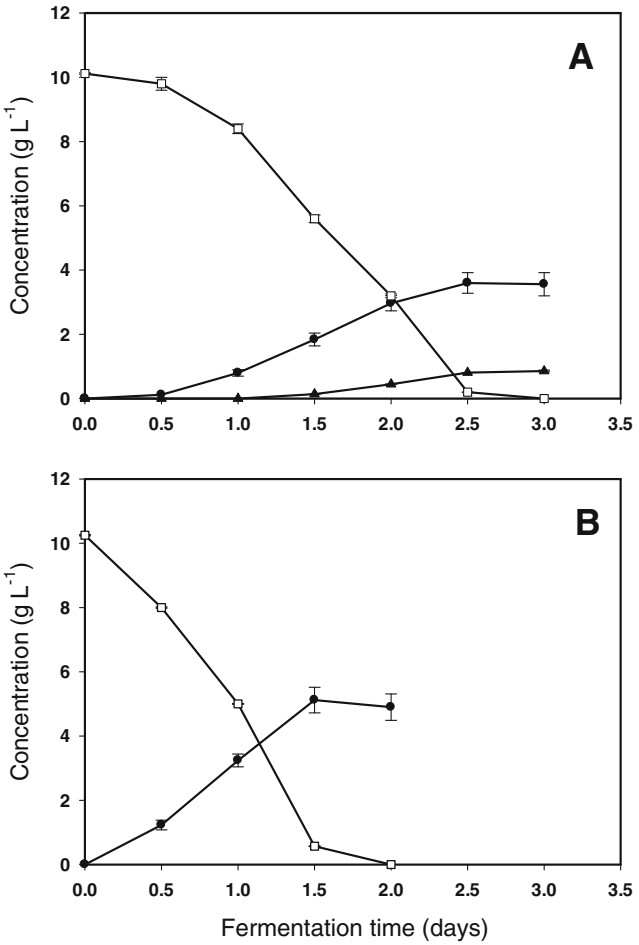


**Fig. 2** Changes in biomass, substrate and product concentration during cultivation of *A. terreus* NRRL 1960 on 10 g D-glucose $L^{-1}$. *Filled circle* fungal dry weight concentration, *open square* D-glucose concentration, *filled triangle* concentration of itaconic acid. **a** Manganese ion-deficient culture (<3 μg/L]. **b** Culture with 0.25 mg $L^{-1}$ manganese ions

(*w*/*v*) (Xu et al. 1989). We therefore tested whether an increase in the D-glucose concentration in the growth medium would also result in a higher molar IA yield. To this end, a series of manganese ion-limited fermentations with different D-glucose concentrations were performed. A plot of the initial concentrations of D-glucose against the specific molar yield of IA shows that this is indeed the case (Fig. 3): maximum $Y_{p/s}$ values of almost 0.90 were obtained at 120 g $L^{-1}$ of D-glucose and were maintained up to 200 g $L^{-1}$. This is close to the theoretical maximum if the carbon spent for biomass is considered and corresponds to the production of 133 g $L^{-1}$ IA from 200 g $L^{-1}$ D-glucose. These concentration values are also close to the maximal solubility of IA in water at the cultivation temperature (1.0623 M $kg^{-1}$ at 306.15 K, equivalent to 138.3 g $L^{-1}$ at 33 °C; Apelblat and Manzurola 1997), indicating that fermentations at still higher concentrations of D-glucose would likely not further increase the final IA concentration.

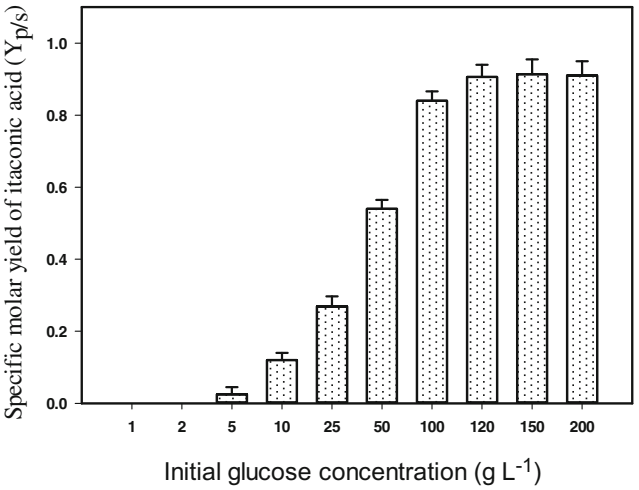


**Fig. 3** Specific molar yield of itaconic acid as a function of the initial D-glucose concentration in manganese ion-deficient cultures of *A. terreus*. For calculation of the yield, D-glucose and IA concentrations in the medium were measured at the time point where maximal IA production was achieved

## The external manganese concentration severely influences the IA yield

Having established conditions leading to IA yields close to the theoretical maximum, we now investigate how manganese ion concentrations would affect the specific IA yield. To this end, the fungus was cultivated on high D-glucose concentration at externally added manganese ion concentrations ranging from 2 μg $L^{-1}$ to 2.5 mg $L^{-1}$. The results (Fig. 4) showed that, similar to *A. niger* citric acid fermentation, the IA yield started to decrease at a manganese ion concentration>5 μg $L^{-1}$. Interestingly, however, and in contrast to *A. niger*, the lowest IA concentration was already obtained at 1 mg $L^{-1}$ manganese ions and was not

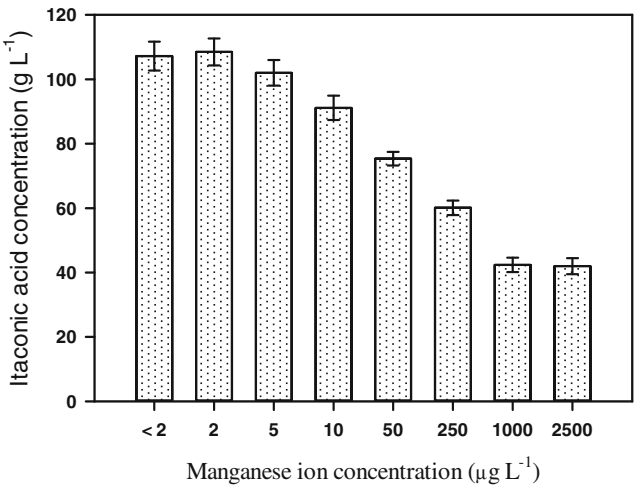


**Fig. 4** Final itaconic acid concentration formed by *A. terreus* on media with different manganese ion concentrations. The initial D-glucose concentration was 180 g $L^{-1}$ in all experiments

affected by a further increase above this level. Under these conditions, a $Y_{p/s}$ of 0.31 was reached.

We also compared the time course of IA production in the presence and absence of manganese ion addition on high D-glucose concentrations (Fig. 5). In the presence of manganese ions, the changes in biomass and residual D-glucose concentrations as well as biomass and IA volumetric yields were similar to those obtained at low D-glucose levels (0.49 and 0.36, respectively). Under manganese ion-deficient conditions, a comparatively restricted biomass formation was noted again with a maximal value of 20.05 g (DCW) $L^{-1}$, which is approximately 60 % lower than under manganese ion sufficiency (Fig. 3). Complete depletion of the D-glucose pool occurred 3 days later. However, IA formation dramatically increased upon the absence of manganese ions in the growth medium: peak mean volumetric yield was 95.3 g $L^{-1}$, corresponding to a specific molar yield of 0.87 and indicating a very high rate of sugar conversion into acid.

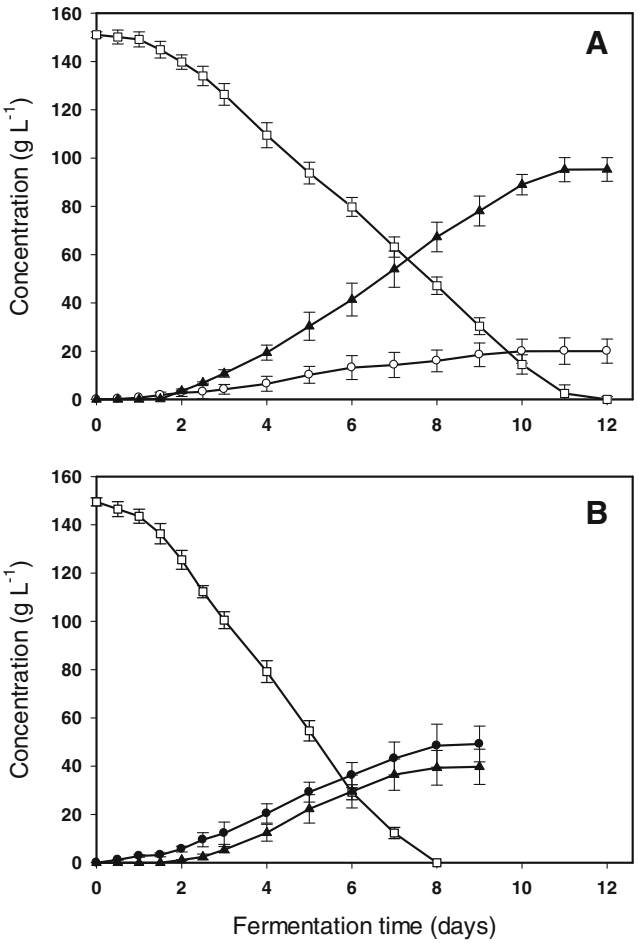


**Fig. 5** Changes in biomass, substrate and product concentration during cultivation of *A. terreus* NRRL 1960 on 150 g D-glucose $L^{-1}$. *Filled circle* fungal dry weight concentration, *open square* D-glucose concentration, *filled triangle* concentration of itaconic acid. **a** Manganese ion-deficient culture (<3 μg $L^{-1}$]. **b** Culture with 0.25 mg $L^{-1}$ manganese ions

### Manganese deficiency affects hyphal morphology

Kisser et al. (1980) described that manganese ion deficiency dramatically altered the morphology of *A. niger* in submerged culture. A similar effect was also observed for *A. terreus*: under manganese ion deficiency, the fungus grew in the form of small and dense pellets that are formed from swollen, short and stubby hyphae, while in the presence of sufficient manganese ions, the cultures overhelmingly consisted of long and smooth hyphae that formed branching filaments or mycelial clumps (Fig. 6). Interestingly, this change was further influenced by the concentration of D-glucose: growth on 15 % (*w*/*v*) D-glucose led to denser mycelial pellets. Measurement of the hyphal diameter throughout the fermentation showed that it was unaffected by the presence of manganese ions on 1 % (*w*/*v*) D-glucose, whereas it was strongly increased on 15 % (*w*/*v*) D-glucose, but remained significantly smaller in the case of manganese ion deficiency (Table 2).

## Discussion

The results described above show conclusively that IA can be produced by *A. terreus* in the same high molar yields ($Y_{p/s}$) as citric acid by *A. niger*, if the manganese ion concentration in the medium is kept below 5 μg/L and the initial sugar concentration is 100 g $L^{-1}$ or higher. Thus, from the point of improving the production process for itaconic acid, there is no need to transfer the respective genes necessary for itaconic acid biosynthesis and export to *A. niger* (Blumhoff et al. 2013; Li et al. 2013; Van der Straat et al. 2014). This does not mean that the conversion of *A. niger* into an IA producer would not be scientifically interesting, but we would strongly suggest to perform such investigations also under the conditions known to lead to high yields (Karaffa and Kubicek 2003; Kubicek et al. 2010).

A detailed reading of the available literature on IA production by *A. terreus* revealed that none of the studies attempted to remove cations from the sugar solution, which—even when analytical grade is used—introduced a concentration of manganese ions that decreased the IA yield as shown by us here. Interestingly, in some studies where an optimization of the (not decationized) medium was performed, the authors reported on an IA yield increasing effect by raising the concentration of calcium or copper ions (Kuenz et al. 2012; Hevekerl et al. 2014). Copper ions have been shown to antagonize the effect of manganese ions (Schweiger 1961), probably by inhibiting the transport of manganese ions (Hockertz et al. 1987; Netik et al. 1997). So these effects could in fact be due to unintended manipulation of the availability of manganese ions.

Because decationization of the medium is not always a convenient process for medium preparation, several other means of antagonizing the effects of manganese ions on citric acid fermentation by *A. niger* were developed that included

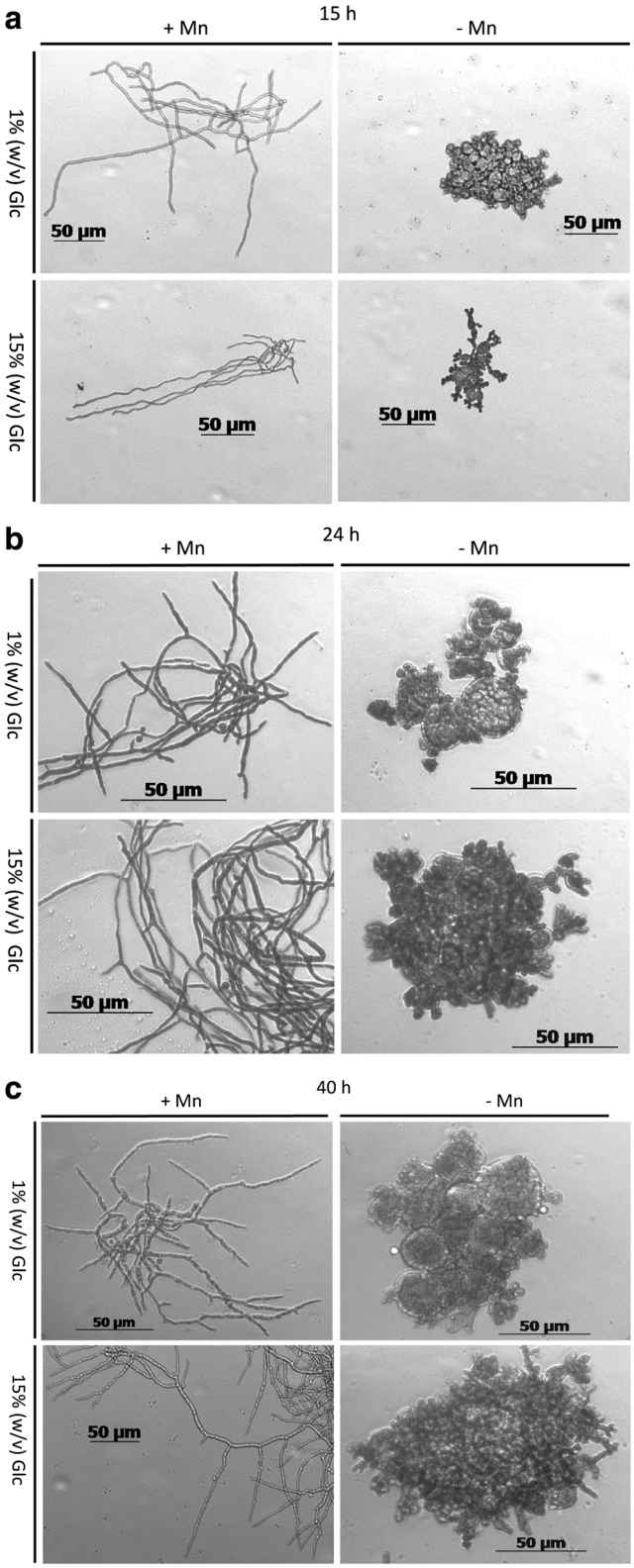


**Fig. 6** Microscopic images of *A. terreus* cultures grown under manganese ion limitation (concentration<3 µg $L^{-1}$, indicated as *− Mn*) and manganese ion sufficiency (concentration 0.25 mg $L^{-1}$, indicated as *+Mn*), both at 1 % (*w*/*v*) or 15 % (*w*/*v*) initial D-glucose concentration. **a**–**c** Images taken at cultivation times of 15, 24 and 40 h, respectively

**Table 2** Mean cell diameter of *A. terreus* cultures determined by quantitative image analysis at different fermentation time-points. Cultures grown under manganese ion limitation (concentration< 3 µg $L^{-1}$, indicated as '− Mn') and manganese ion sufficiency (concentration 0.25 mg $L^{-1}$, indicated as '+Mn'), both at 1 % (*w*/*v*) or 15 % (*w*/*v*) initial D-glucose (Glc) concentration

| | 15 h | 24 h | 40 h |
|---|---|---|---|
| + Mn, 1 % Glc | 2.57±0.51 | 2.80±0.53 | 3.16±0.64 |
| + Mn, 15 % Glc | 3.01±0.62 | 2.50±0.54 | 2.67±0.62 |
| − Mn, 1 % Glc | 8.16±1.98 | 13.98±4.46 | 16.07±6.07 |
| − Mn, 15 % Glc | 8.23±1.78 | 10.32±2.27 | 8.36±3.56 |

addition of copper (up to 50 mg $L^{-1}$; see above), low molecular weight alcohols, lipids and tertiary amines (Moyer 1953; Röhr et al. 1996). It will be interesting to test whether their addition also increases the IA yield by *A. terreus* in non-decationized media.

The similarity of effects of manganese ions on citric and IA production also provides the chance to re-evaluate the different biochemical consequences that have been attributed to manganese ion deficiency: the eldest one is the hypothesis that the flux rate through glycolysis may be stimulated because manganese ion deficiency causes an enhanced pool of $NH_4^+$ ion which is capable of antagonizing the inhibition of PFK1 by citrate (Schreferl et al. 1986). Enhanced intracellular concentrations of citrate under manganese ion deficiency have been shown (Habison et al. 1983), and their localization also in the cytosol is likely because citrate has to pass through it before being transported out of the cell. During IA accumulation, however, it is itaconate that accumulates in the cytosol, and the concentration of citrate should not be significantly higher than under conditions not producing IA. It is not known whether itaconate inhibits PFK1, but it has been shown to mimick the inhibition of PFK2 by phosphoenolpyruvate (PEP) (Sakai et al. 2004). Since PEP is also an inhibitor of PFK1, we speculate that also IA has an inhibitory effect of glycolytic flow at PFK1. In fact, transformation of *A. terreus* with a gene encoding a citrate-resistant fragment of PFK1 stimulates the rate of production and final titer of itaconic acid (Tevz et al. 2010), indicating the importance of the flux rate through glycolysis also for IA fermentation.

Another explanation for the effect of manganese ion deficiency on the accumulation of citric acid was the necessity of manganese ions to form complexes with citrate that are then the substrate for re-uptake into the cell and thus reduce the accumulation of citric acid outside of the cell (Netik et al. 1997). Dicarboxylic acids are also capable of forming complexes with divalent metal ions, but lack the OH– and one COO– group that participate in citrate, and the dissociation constants for complexes between itaconic acid and manganese are not known (Matzapetakis et al. 2000). Also, the properties of the itaconic acid permease have not yet been studied.

Yet a third effect of manganese ion deficiency in *Aspergillus* is very well documented, i.e. its effect on cell wall formation and consequently mycelial morphology (Detroy and Ciegler 1971; Zonneveld 1975; Kisser et al. 1980; Dai et al. 2004). During submerged cultivation of *A. niger* and *A. terreus* (as shown in this study), this is reflected in the formation of short, bulbeous and branched hyphae that form very small and tight pellets. These pellets result in a considerably improved rheology of the culture broth and thus increased mass transfer (Schweiger 1961). Also, the oxygen concentration in fungal pellets decreases rapidly with the depth>0.8 mm (Michel et al. 1992). Since high oxygen tension in the medium is critical for achieving high titers of citric and itaconic acid (Kubicek et al. 1980; Park et al. 1993), it is likely that manganese ion deficiency has a major role in guaranteeing it. Gyamerah (1995) showed that pellets smaller than 1 mm in size are enhanced producers of itaconic acid.

In addition to the critical effect of manganese ions, we have also shown that optimal molar yields of IA are only obtained at D-glucose concentrations >100 g $L^{-1}$. We should like to stress here that this is not simply a "more substrate leads to more product" finding because the final concentration of IA does not linearly increase with the D-glucose concentration. In fact, at 50 g $L^{-1}$, the IA yield is 0.57 moles/mole glucose, whereas it is 0.82 moles/mole glucose at 100 g $L^{-1}$ (cf. Fig. 3). It is important to note in this context that yields up to 0.66 moles/mole D-glucose can be obtained without the anaplerotic carbon dioxide fixation by the cytosolic pyruvate carboxylase (Fig. 1), whereas any higher yield depends on its operation. In *A. niger*, increasing the sugar concentration has been shown to stimulate the formation of pyruvate carboxylase (Feir and Suzuki 1969; Peksel et al. 2002), and our data suggest that a similar mechanism may exist in *A. terreus*.

Concluding, we have demonstrated that a simple manipulation of two parameters of the medium, known to be relevant for citric acid production by *A. niger*, also enables *A. terreus* to convert D-glucose to IA at yields approaching the theoretical maximum. We hope that these findings will be used in subsequent studies on IA biosynthesis.

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**Ethical statement** The authors declare no conflict of interest.

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