FINAL REPORT

Itaconic acid overflow in *Aspergillus terreus*: how fermentation technology reflects metabolic needs (KH 129602)

Introduction, background

Itaconic acid (2-methylenesuccinic acid, 1-propene-2-3-dicarboxylic acid; $C_5H_6O_4$) is an unsaturated, weak dicarboxylic acid (pK_a = 3.83 and 5.41), discovered in 1837 as a thermal decomposition product of citric acid. At room temperature it is a white solid that is soluble in water, ethanol, and acetone. The presence of the conjugated double bond of the methylene group allows polymerization both by addition and condensation. Esterification of the two carboxylic groups with different co-monomers is also possible (Kuenz et al. 2012). These diverse properties have led to a variety of applications in the pharmaceutical, architectural, paper, paint, and medical industries such as as plastics, resins, paints, synthetic fibers, plasticizers, detergents. Recently, itaconic acid applications have penetrated the dental, ophthalmic and drug delivery fields (Hajian and Yusoff 2015). Itaconic acid polymers could even replace the petroleum-based polyacrylic acid, which has a multi-billion dollar market. Not surprisingly, the US Department of Energy in 2004 assigned itaconic acid as one of the top 12 most promising building block chemicals for bio-based economy. However, to fulfill its potential, the selling price of itaconic acid – currently at approximately $2.0/kg – must drop considerably. Many of the plants in developed countries have therefore relocated to China, which now accounts for the overwhelming majority of global itaconic acid production, estimated at 42.000 tons per year (Geiser et al., 2016).

Early observations about fungal itaconic acid accumulation date back to the 1930s, while the first fermentation technology – already employing *Aspergillus terreus*, the species used for industrial itaconic acid production ever since – was patented in the next decade (Kane et al. 1945). Production is exclusively performed by submerged, fermentation in batch mode (Cavallo et al. 2017). Regarding scale, vessel types, inoculation protocols, critical process parameters, medium composition, the upstream of the *A. terreus* itaconic acid fermentation is much similar to the *A. niger* citric acid production. Itaconic acid is thus produced on molasses or hydrolyzed starch, applied at very high (> 12%, w/v) concentrations. The process is strongly aerobic, and extremely sensitive to the presence of Mn(II) ions. However, excess of Cu(II) ions results in mitigation of the deleterious effect that Mn(II) ions exert on the formation of itaconic acid (Saha and Kennedy, 2019a).

The biosynthetic pathway of itaconic acid resembles that of citric acid, the latter acid being a direct precursor of the former. The only difference is that citric acid in *A. terreus* is further metabolized via *cis*-aconitate to itaconate by *cis*-aconitate decarboxylase. Citrate is likewise synthesized from oxaloacetate and acetyl-CoA, while oxaloacetate is synthesized from pyruvate by anaplerotic CO_2 fixation in the cytosol. It is then shuttled into mitochondria by a specific antiporter in exchange for *cis*-aconitate. Itaconic acid – formed upon *cis*-aconitate decarboxylation – is secreted out of mycelia by a specific cell membrane transporter. Genes encoding these three enzymes (and a fourth one encoding a transcription factor) constitute the „itaconate gene cluster” in the *A. terreus* genome.

Hypothesises to test

On technical scale, itaconic acid is produced from glucose-containing complex carbon sources. However, stiff competition with food applications keeps the fermentation industry searching for cheap, renewable raw materials to be utilized. Hence, research interests over the more efficient utilization of non-food, lignocellulosic plant biomass are soaring for a long time now (Cunha da Cruz et al., 2018). Lignocellulose is a complex polymer of hexose and pentose monomers, whereby D-xylose is the most abundant pentose. However, D-glucose and D-xylose as well as D-xylose and L-arabinose partially interfere with each other's uptake and metabolism, and thus investigation of their combined conversion will only result in scientifically valid data if the metabolism of these sugars in the absence of the others is understood first. Since itaconic acid in *A. terreus* is formed by the same metabolic pathway as citric acid in *A. niger*, it was not unreasonable to assume that the physiological requirements for itaconic production from D-xylose (and consequently the fermentation parameters) would be similar to those on D-glucose. There is a major difference, however, because the fungal catabolism of D-xylose occurs via the pentose catabolic pathway (Khosravi et al. 2018) and only at later stages feeds its intermediates into glycolysis.

In this project we have therefore tested whether two landmark nutritional requirements of the *A. terreus* itaconic acid overflow on D-glucose – Mn(II) ion deficiency and high concentration of the carbon source – also occur in a similar fashion on D-xylose as a sole source of carbon, and whether their respective optimization would give as high molar yields from D-xylose as from D-glucose. Subsequently, the ability of Cu(II) ions to alleviate the negative effect of Mn(II) ions on itaconic acid fermentations have been tested on carbon sources that constitute lignocellulose – that is, D-xylose, L-arabinose, D-glucose and D-fructose.

Results and Discussion

Itaconic acid production from D-xylose is stimulated by manganese deficiency

D-xylose is first catabolized to xylulose-5-phosphate, which enters the pentose phosphate pathway. The correspondingly arising intermediates fructose-6-phosphate and glyceraldehyde-3-phosphate would then enter glycolysis. If we assume that the itaconic acid biosynthetic pathway would further commence by anaplerotic formation of oxaloacetate by pyruvate carboxylase, the maximal amount of itaconic acid that can be obtained from D-xylose is 0.83 ($Y_{p/s}$; moles/moles) or 72 g per 100 g of D-xylose.

We have reported previously that production of itaconic acid from D-glucose requires a strict limitation of Mn(II) ions in the medium (Karaffa et al., 2015). We therefore tested whether this is also the case for itaconic acid production from D-xylose at a concentration of 50 g/L. **Figure 1** shows that the highest molar yield ($Y_{p/s}$ = 0.63) of itaconic acid was obtained at cultivation below 3 µg/L Mn(II), which represents 75% of the theoretical maximum molar yield. Increasing the concentration of Mn(II) ions up to 100 µg/L results in an approximately 50 % reduction of the itaconic acid yield, which is comparable to what has been published for D-glucose (Karaffa et al., 2015). High production of itaconic acid from D-xylose therefore also requires a deficiency of Mn(II) in the medium. This finding support the interpretation that the effect of Mn(II) ions is independent of the carbon source and is rather due to an interaction either with transport and/or improved rheology via the compact pellet morphology, which in turn results in increased oxygen transfer throughout the fermentation.

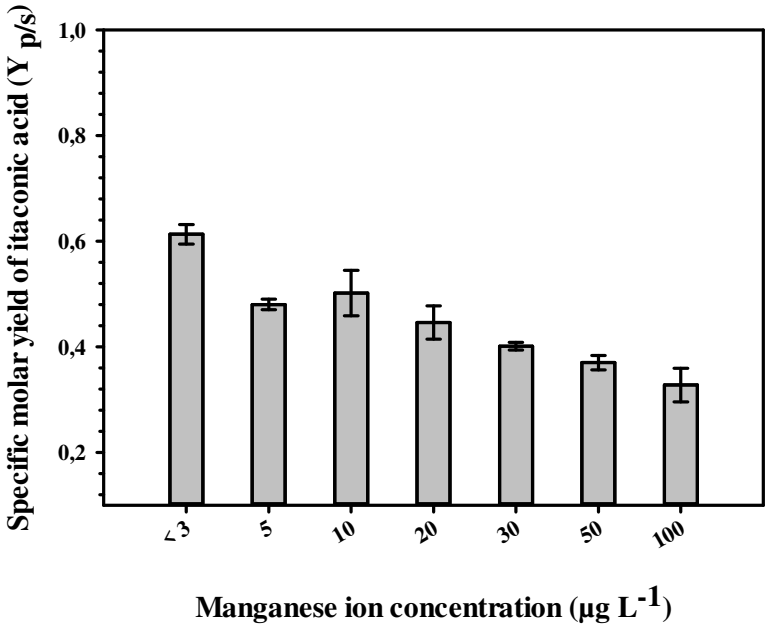

Figure 1. Final specific molar yield of itaconic acid in submerged *Aspergillus terreus* NRRL 1960 cultures. The initial D-xylose concentration was 50 g/L in itaconic acid-producing medium supplemented with different concentrations of manganese(II) ions.

The morphology of D-xylose grown A. terreus is dependent on the concentration of Mn(II)

The morphology of a fungal culture has an important impact on its behaviour in submerged cultivation because it influences the surface available for nutrient transport and the viscosity of the medium. A deficiency in manganese ions is known to change the morphology of *Aspergillus spp*. from smoothly filamentous to stubbed, swollen, highly branched and even yeast-like hyphae (Barnett and Lilly, 1966; Clark et al., 2016). In agreement with this, the morphology of *A. terreus* at Mn(II) concentration <3 µg/L was represented by yeast-like forms and loose cell clumps. Formation of hyphae occurred only over 5 µg/L. In line with this, the cell diameters decreased gradually and significantly with increasing Mn(II) concentrations in the growth medium, the same behaviour also observed with the itaconic acid yield. Summarizing, similarly to the case on D-glucose, morphology of D-xylose grown *A. terreus* cultures – quantified as cell diameter – is extremely sensitive to the changes in the Mn(II) ion concentration in the medium, even within a range considered entirely suboptimal for growth (from <3 to 10 µg/L).

The highest molar yield of itaconic acid from D-xylose is already reached at 50 g/L

The second critical parameter for itaconic acid production from D-glucose is the concentration of the carbon source, and maximal yields are obtained only at concentrations over 120 g/L (Karaffa et al., 2015). We therefore tested whether the optimal concentration of D-xylose would also be in this range. The results are shown in **Figure 2**: there is indeed a steady increase in itaconic acid formation when the D-xylose concentration is raised, resulting a plateau already at in a maximal concentration of 54 g/L itaconic acid at 11% (w/v) xylose. This corresponds to a molar yield of 55% and represents 73% of the theoretical maximum (83%). The maximal and overall volumetric productivity under these conditions were 0.37 and 0.23 g itaconic acid/L/h, respectively. In contrast to the findings with D-glucose, this highest molar yield (63%) is

already reached at 5% (w/v) D-xylose. The $Y_{p/s}$ at 11 % (w/v) is lower than at 5% (w/v), although the difference is statistically not significant ($p = 0.32$). However, irrespective of the initial concentration of D-xylose, kinetics of four fundamental fermentation parameters – pH, and concentration of biomass, D-xylose and itaconic acid – were qualitatively similar.

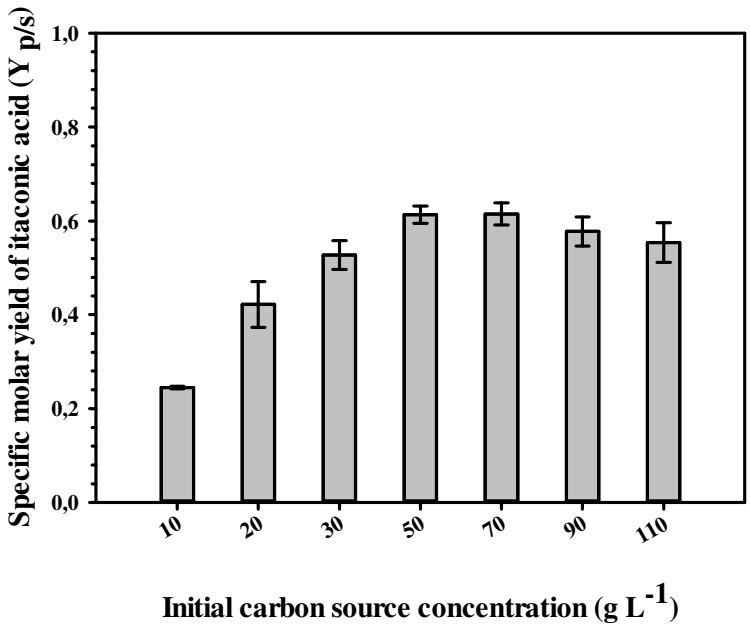

Figure 2. Specific molar yield of itaconic acid at different initial D-xylose concentrations in manganese(II) ion limited cultures of *Aspergillus terreus*. Yields were calculated from the consumed D-xylose and maximal achieved IA concentrations.

Biomass and by-product formation during itaconic acid production on D-xylose

The conversion of only about 50 % of the available and fully consumed D-xylose into itaconic acid raises the question about the fate of the remaining carbon. One point to be considered here is the release of 1 mole of carbon dioxide per 1 mole of itaconic by the *cis*-aconitate decarboxylase (CAD) reaction. A calculation shows that this accounts for up to 18.2 g/L on 11% (w/v) xylose. We also checked for the presence of xylitol, which is a common by-product of fungal growth in D-xylose (Weyda et al., 2014), but found only very low concentrations (~ 0.4 g/L; corresponding to ~ 8 mmoles/mole D-xylose). We further detected small amounts of α-ketoglutarate in the medium and they increased from 0.3 to 1.2 g/L when the concentration of D-xylose was raised from 5 to 11% (w/v), which resembles 11 mmoles/mole D-xylose. No other extracellular carbon components were detected. Consequently, we can rule out the formation of by-products as a reason for the gap between the theoretical and the achieved maximal molar yield of itaconic acid.

Because of these findings, the remaining gap in the carbon used and products found must be accounted for by biomass formation. We found that under optimized itaconic acid producing conditions – that are obviously suboptimal for growth – the biomass yield (g per g carbon source present) for D-xylose was 0.1 at 5% (w/v) and 0.08 at 11% (w/v) initial concentrations. In contrast, the biomass yield was reported to be 0.14 (w/w) for D-glucose (Molnár et al., 2018) under otherwise identical conditions. However, when the biomass yield is

calculated only on the basis of the remaining carbon (i.e. after eliminating the carbon needed for itaconic acid and side-product formation), cultivation of *A. terreus* at 1% (w/v) D-xylose led to a biomass yield of $Y_{x/s}$ = 0.45. This specific biomass yield gradually declines with increasing D-xylose concentration: at 11% (w/v), the itaconic acid concentration formed is 54 g/L, the carbon dioxide removed by CAD is 18.2 g/L, and thus the $Y_{x/s}$ is only 0.23. We therefore concluded that itaconic acid is indeed the only product of cultivation on D-xylose, and its lower molar yield is due to a high carbon demand for biomass production. Our data and these considerations therefore strongly suggest that an increase in the yield coefficient of biomass formation from D-xylose would be a tool for further increasing the yield of itaconic acid from D-xylose.

Copper(II) ion tolerance of Aspergillus terreus depends on the carbon source and the concentration of manganese ions

A synthetic growth medium optimized for itaconic acid production (Kuenz et al., 2012) was used to test copper tolerance of A. terreus NRRL1960 using two hexoses (D-glucose, D-fructose) and two pentoses (D-xylose, L-arabinose) as carbon sources. Each carbon source was used at a concentration that allowed the highest itaconic acid yield: 120 g/L for D-glucose and D-fructose, 80 g/L for L-arabinose and 50 g/L for D-xylose. The default Cu(II) concentration that allowed the highest growth rate was 3.3 mg/L for each carbon source tested. The Mn(II) ion concentration was set either at 1.5 µg/L – which is growth limiting but favours itaconic acid production – or 300 µg/L, which is optimal for biomass formation (Saha and Kennedy, 2019b).

In the presence of 300 µg/L manganese ions, the addition of increased concentrations of copper ions started to decrease the specific biomass yield ($Y_{x/s}$) from the carbon source at concentrations > 50 mg/L (with the exception of L-arabinose where the decrease was only apparent at > 100 mg/L). Yet the half-maximal lethal concentrations (LD_{50}) of copper ions on the two hexoses were higher (close to 1 g/L) than on the two pentoses (around 0.75 g/L). In the presence of 1.5 mg/L manganese ions, however, the decrease in biomass yield started already at copper concentrations of > 75 mg/L on the two hexoses and at >25 mg/L with the two pentoses. In agreement with these findings, the half-maximal lethal concentration of copper ions was around 100 mg/L for the two hexoses, and 78 mg/L for D-xylose. The LD_{50} value of Cu(II) for L-arabinose was even only 48 mg/L. The sensitivity of *A. terreus* to Cu(II) ions thus appears to depend on the concentration of manganese ions as well as on the growth substrate, particularly at limiting concentrations of Mn(II).

The germination of conidiospores tolerated much higher concentrations of copper – up to 3 g/L for hexoses in the presence of 300 µg/L manganese ions – but otherwise showed the same trend as the specific biomass yield, the sensitivity being higher at limiting manganese concentrations and during growth on pentoses.

The concentrations of Cu(II) and Mn(II) influence the itaconic acid yield in a carbon source dependent manner

Under fully optimized fermentation conditions (initial substrate concentration, culture broth pH, Mn(II) limitation, high DO) *A. terreus* NRRL1960 is capable of converting >80% of the available D-glucose, on a molar basis (Yp/s), into itaconic acid (Karaffa et al., 2015). Slightly lower specific yields were achieved on D-fructose, whereas significantly lower yields were

attained on D-xylose and particularly on L-arabinose. Specific molar itaconic acid yields were always highest at a Cu(II) concentration of 3.3 mg/L. On the two hexoses, the lowest yields were observed at the lowest of the Cu(II) concentrations tested (0.01 mg/L). On D-glucose, D-fructose and D-xylose, the molar itaconic acid yield ($Y_{p/s}$) decreased only slightly at copper concentrations higher than 3.3 mg/L, which was accompanied by an increased yield of itaconic acid per biomass unit ($Y_{p/x}$). However, unlike hexose-grown cultures, the pentose-grown cultures did not form any itaconic acid at a Cu(II) concentration of ≥75 mg/L. Finally, the generally low itaconic acid yields on L-arabinose did not seem to variate with the copper concentration in the growth medium.

Mn(II) inhibition of itaconic acid formation is mitigated by Cu(II) ions in a carbon source-dependent manner

Increasing the extracellular Mn(II) ion concentrations in the medium significantly lowered the maximal specific molar yield of itaconic acid on all four carbon sources. At 3.3 mg/L Cu(II), i.e., the concentration conducing optimal itaconic acid production under conditions of Mn(II) paucity, and 300 µg/L Mn(II), the molar yield decreased by 21% on D-glucose and by 16% on D-fructose relative to the best conditions for itaconic acid production. Lowering the Cu(II) ion concentration down to 0.01 mg/L did not change biomass production of the cultures but significantly decreased molar itaconic acid yield. However, increasing the Cu(II) concentration gradually increased the specific molar itaconic acid yield. On D-glucose, essentially the same itaconic acid yields could be reached at 1.5 µg/L Mn(II) / 3.3 mg/L Cu(II) and at 300 µg/L Mn(II) / 300 mg/L Cu(II). Similarly, molar yields could be restored to over 90% of the optimal yield on D-fructose by increasing the amount of Cu(II) ions by two orders of magnitude to counteract the 200-fold excess of Mn(II). However, contrary to the situation on the two glycolytic hexoses, the inhibitory effect of Mn(II) ion sufficiency on itaconic acid production was not fully alleviated by an excess of Cu(II) ions in the case of either of the pentoses as the growth substrate. On D-xylose, specific molar itaconic acid yield increased with rising copper ion concentrations, but only to half of the maximal yield obtained at 1.5 µg/L Mn(II), whereas no attenuation could be observed on L-arabinose as the carbon source.

The ratio of manganese and copper ions concentrations affects fungal morphology

Under conditions conductive to itaconic acid production, the morphology of D-glucose-grown *A. terreus* cultures is characterized by small, compact pellets and yeast-like cells, rather than elongated hyphae (**Figure 3**). They are characterized by increases in cell diameter and decreases in pellet diameters. These two measurable parameters were therefore assessed for variation during the course of the fermentations. On D-glucose at 1.5 µg/L Mn(II) ions, the average cell diameter in the 24-h old cultures was 2.41 ± 0.58 µm when 0.01 mg/L Cu(II) ions were present in the culture broth, and displayed a continuous increase up until a maximum diameter was observed at 25 mg/L. Above 25 – 50 mg/L Cu(II) the trend reversed, and the average cell diameter were not significantly lower (3.57 ± 1.04) at 250 mg/L Cu(II) than they were at 0.01 mg/L. The difference between the most extreme cell diameters observed was over four-fold, and the largest cell diameters were observed in the cultures with the highest molar itaconic acid yields. These trends were similar at each time-point tested, from a day after inoculation until

carbon source exhaustion, although the span between the two most extreme cell diameter values decreased with the culture age. Pellet sizes followed an opposite pattern in that the pellets were at their largest at 0.01 mg/L Cu(II), and sharply decreased in diameter with increasing Cu(II) concentrations. The pellets were at their smallest at 50 mg/L Cu(II).

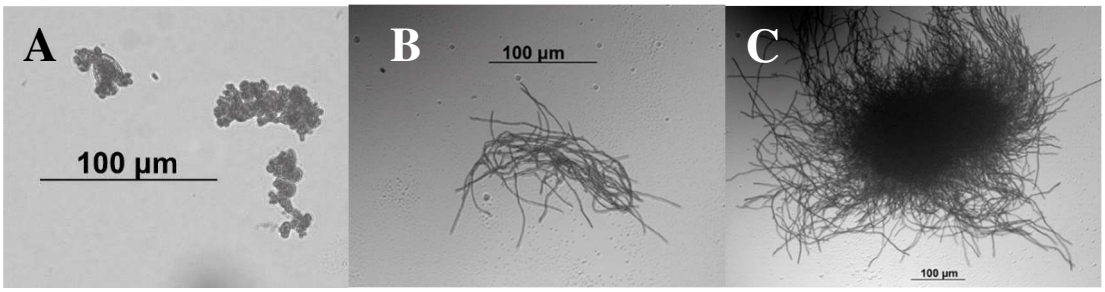

Figure 3. Definition of the three typical morphological forms of *Aspergillus terreus* NRRL 1960 (glucose-grown cultures). Panel A: "yeast-like", i.e., swollen globular cells; Panel B: filamentous hyphae; Panel C: pellet.

Fungal morphology was fundamentally different in the presence of sufficient Mn(II) ions in the growth medium, 300 µg/L. Maximal average cell diameters from early time-point samples were either smaller or similar than the later ones, and the diameters were generally lower than those measured under Mn(II) limitation. Cultures with Cu(II) ion concentrations in the range of 0.01 and 100 mg/L displayed mostly filamentous morphology with average cell diameters less than 2.5 µm. Cultures with copper concentrations > 100 mg/L had increasingly higher cell diameters, particularly in the later stages of cultivation (72, 96, 168 h). At 300 and 400 mg/L Cu(II) ions, average cell diameters were 60 – 70% of the values measured in cultures grown under itaconic acid production conditions. Our data suggest that variation in average cell diameter is correlated with the ratio of manganese(II) and copper(II) ions in the fermentation rather than with the concentration of either of the two cations. Hyphal diameter remained ~ 2 µm during the course of the fermentation as long as the Mn:Cu ratio was higher than 1.2×10^{-3}. However, average diameter of cells – as well as specific molar itaconic acid yields – significantly increased when the Mn:Cu ratio fell between 1×10^{-3} and 0.75×10^{-3}. No such correlation was found in the cultures grown at low Mn(II) ion concentrations.

D-Xylose-grown cultures likewise displayed the typical overflow-associated fungal morphology under manganese limitation, with average cell diameters increasing with the Cu(II) concentration and with the cultivation time. However, formation of pellets with characteristic core region was observed at lower Cu(II) ion concentrations tested: >350 µm pellets at 0.01 mg/L and >250 µm pellets at 1 mg/L, as opposed to the morphology seen at the standard Cu(II) ion concentration of 3.3 mg/L, optimal for itaconic acid yield also on D-xylose. Under Mn(II) ion sufficient conditions, morphology on D-xylose was generally similar to that in D-glucose fermentations. However, no correlation could be observed between any of the morphological parameters investigated and the itaconic acid production measured either on this pentose or on L-arabinose.

Summary

Itaconic acid is used as a bio-based, renewable building block in the polymer industry. It is produced by submerged fermentations of the filamentous fungus *Aspergillus terreus* from molasses or starch, but research over the efficient utilization of non-food, lignocellulosic plant biomass is soaring. An important objective of this project was to test whether the application of two key cultivation parameters for obtaining itaconic acid from D-glucose in high yields – Mn(II) ion deficiency and high concentration of the carbon source – would also occur on D-xylose, the principal monomer of lignocellulose. To this end, a carbon and energy balance for itaconic acid formation was established, which is 0.83 moles/mole D-xylose. The effect of Mn(II) ions on itaconic acid formation was similar to that on D-glucose and maximal yields were obtained below 3 µg/L Mn(II) ions, which were, however, only 0.63 moles of itaconic acid per mole D-xylose. In contrast to the case on D-glucose, increasing D-xylose concentration over 50 g/L did not change the above yield. By-products such as xylitol and α-ketoglutarate were found, but they cumulatively remained below 2% of the concentration of D-xylose. Mass balance of the fermentation with 110 g/L D-xylose revealed that >95% of the carbon from D-xylose was accounted as biomass, itaconic acid and the carbon dioxide released in the last step of itaconic acid biosynthesis. These data show that the efficiency of biomass formation is the critical parameter for itaconic acid yield from D-xylose under otherwise optimal conditions. In the presence of higher external Mn(II) concentrations itaconic acid yield decreases and biomass formation is favored, but this could be mitigated by increasing the Cu(II) concentration in the medium. *A. terreus* displayed a very high tolerance to Cu(II) which, however, decreased when Mn(II) availability became increasingly limiting. High (>75%) specific molar itaconic acid yields always coincided with an "overflow-associated" morphology, characterized by small compact pellets (<250 µm diameter) and short chains of "yeast-like" cells that exhibit increased diameters relative to the elongated cells in growing filamentous hyphae. At low concentrations (≤1 mg/L) of Cu(II) ions, manganese deficiency did not prevent filamentous growth. Mycelial- and cellular morphology progressively transformed into the typical overflow-associated one when external Cu(II) concentrations increased, irrespective of the available Mn(II). Our results indicate that copper ions are relevant for overflow metabolism and should be considered when optimizing D-xylose based itaconic acid fermentation in *A. terreus*.

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1) **Karaffa L.**, Kubicek C.P. (2019): Citric acid and itaconic acid accumulation: variations of the same story? *Applied Microbiology and Biotechnology,* 103: 2889-2902.
Impakt faktor: 3.670 (Q1)

2) Kolláth I.S., Molnár Á.P., Soós Á., Fekete E., Sándor E., Kovács B., Kubicek C.P., **Karaffa L.** (2019): Manganese deficiency is required for high itaconic acid production from D-xylose in *Aspergillus terreus*. *Frontiers in Microbiology*, 10: 1589.
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3) Sándor E., Kolláth I.S., Fekete E., Bíró V., Flipphi M., Kovács B., Kubicek C.P., **Karaffa L.** (2021): Carbon-source dependent interplay of copper and manganese ions modulates the morphology and itaconic acid production in *Aspergillus terreus*. *Frontiers in Microbiology*, doi: 10.3389/fmicb.2021.680420. **Impakt faktor: 4.235 (Q1)**

Scientific papers related to this project (grant number indicated)

1) Kavalecz N., Ág N., **Karaffa L.**, Scazzocchio C., Flipphi M., Fekete E. (2019): A spliceosomal twin intron (stwintron) participates in both exon skipping and evolutionary exon loss. *Scientific Reports*, 9: 9940. **Impakt faktor: 4.011 (D1)**

2) Fejes B., Ouedraogo, J-P., Fekete E., Sándor E., Flipphi M., Soós Á., Molnár Á.P., Kovács B., Kubicek C.P., Tsang A., **Karaffa L.** (2020): The effects of external Mn^{2+} concentration on hyphal morphology and citric acid production are mediated primarily by the NRAMP-family transporter DmtA in *Aspergillus niger*. *Microbial Cell Factories*, 19: 17.
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3) Ág N., Kavalecz N., Pénzes F., **Karaffa L.**, Scazzocchio C., Flipphi M., Fekete E. (2020): Complex intron generation in the yeast genus *Lipomyces*. *Scientific Reports*, 10: 6022.
Impakt faktor: 3.998 (Q1)