Short thesis for the degree of doctor of philosophy (PhD)

Aspergillus terreus itaconic acid fermentation: connections between physiology and technology

by István Sándor Kolláth

Supervisor: Dr. Levente Karaffa

UNIVERSITY OF DEBRECEN

Doctoral School of Chemistry

Debrecen, 2022

1. Introduction and objectives

Itaconic acid is an organic acid, which mainly produced via submerged fermentation by employing *Aspergillus terreus* filamentous fungus. Itaconic acid, and first of all its derivatives have wide range of applications (superabsorbents, synthetic latex, detergents, coatings, paints, lacquers), and more and more possibilities come to light (drug carriers, acrylic glass, artificial dental cements) year by year. If the production costs of polyitaconic would have to decline, then it will able to replace the petrochemical based polyacrylic acid. Many studies aim to maximise the yield of the fermentation or find cheaper alternative carbon sources.

During my doctoral scholarship period, my fist objective was to compare the D-glucose and D-xylose based fermentations regarding to itaconic acid production. The manganese sensitivity of the glucose based itaconic fermentations had revealed just before the beginning of my scholarship. We aimed to expand the investigation to xylose based fermentations, in order to to create a model system for itaconic acid fermentations on plant biomass hydrolysates.

Further objective was to find any physiological solution to antagonize the negative effects of the manganese ions in the fermentation medium. Based on the scientific literature, the variation of the phosphate and Cu(II) content could provide the most promising result. In the

experimental design phase, we routinely collected data from *Aspergillus niger* citric acid fermentation literature, which far better documented, technologically and biochemically very similar method to the itaconic acid production. The experiments were done in shaked flask fermentations.

During our research, we always payed prominent attention to the fungal morphology. Although, some publications were showed the importance of the link between the itaconic acid production and the morphology of the *A. terreus*, the available information is still scarce even nowadays.

2. Material and methods

2.1 Fungal strain and cultivation conditions

Aspergillus 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, Netherlands). The strain was stored in 1:1 glycerine-spore wash solution (9 g L^{-1} NaCl, and 20 drops L^{-1} Tween 80) cryoprotectant mixture containing stocks at -80°C. The refreshing of the strain was done every half a year. CSL+Glü agar was used for cultivating the strain (**Table 1**). The plates were incubated for 5-7 days at 37°C until the proper sporulation of the cultures. After the incubation period, the agar plates were sealed with Parafilm and stored at +4°C up to one month.

Table 1 Composition of the CSL+Glü agar for *A. terreus* cultivations. D-glucose was sterilized separately and later added to the media in laminar flow box.

Component	**Concentration (g L^{-1})**
D-glucose	20
corn steep liquor	20
NaCl	20
Agar	20
pH= 5	

2.2 Control of the manganese ion concentration

To control the concentration of Mn^{2+} ions in the growth medium, the given carbon source was dissolved in distilled water and passed through a column (440 x 45 mm) of Dowex 50 W-X8 (100/200) cation exchange resin. All components were added to this solution from sterile stock solutions. The final Mn(II)-ion concentration was adjusted with $MnCl_2$ x 4 H_2O.

The Schott bottles and Erlenmeyer shake flasks were filled with 0.1 M HCl and allowed to stand for 3-4 hours. The equipment were flushed with cation changed water afterwards.

2.3 Fermentation

Shake-flask cultivations were performed in 500-mL Erlenmeyer flasks (VWR International Kft., Debrecen, Hungary) with 100 mL medium incubated at 33 °C in rotary shaker (Infors AG, Basel, Switzerland) operating at 300 rpm, a rotation rate proven to provide sufficient aeration for itaconic acid overflow. The initial pH was set at 3.0 with 3 M HCl. Chemically defined minimal medium (optimised by Kuenz in 2012) was used in the experiments (**Table 2**), unless if it is stated otherwise.

Table 2 Composition of the *A. terreus* itaconic acid production medium.

Component	Concentration (g L^{-1})
KH_2PO_4	0.1
NH_4NO_3	3
$MgSO_4 \times 7\ H_2O$	1
$CaCl_2 \times 2\ H_2O$	5
$FeCl_3 \times 6\ H_2O$	1.67×10^{-3}
$ZnSO_4 \times 7\ H_2O$	8×10^{-3}
$CuSO_4 \times 7\ H_2O$	15×10^{-3}
pH= 3.00	

Copper-, iron and zinc stock solutions were filtere sterilized separately and later added to the media in laminar flow box.

The initial carbon source concentration was varied experiment by experiment in the following ranges:

-D-glucose: 10-120 g L^{-1}

-D-xylose: 10-110 g L^{-1}

-D-fructose: 50-120 g L^{-1}

- L-arabinose: 50-120 g L^{-1}

All carbon sources were sterilized separately. All chemicals used were of analytical grade and purchased from Sigma-Aldrich (Budapest, Hungary).

2.4 Analytical methods

2.4.1 Sample preparation

The fungal mycelia were separated in Eppendorf tubes via centrifugation. The supernatant was used for HPLC and ICP-MS measurements. The samples were frozen for later use at -20°C. The frozen samples were reheated to the temperature of the fermentations before dilution.

2.4.2 Determination the carbon source and itaconic acid concentration

The concentrations of the sole carbon source, itaconic acid in the growth media were determined by high-pressure/performance liquid chromatography (HPLC). For the measurements Agilent 1260 Infinity II (Agilent Technologies, Santa Clara, USA) and Gilson 305 (Gilson, Middleton, USA) instruments were used, equipped with a proton exchange column (Bio-Rad Aminex HPX-87H^+) at T = 55 °C, using isocratic elution with 10 mM H_2SO_4 and refractive index (RI) detection. For xylitol determination, another ion exchanger column (Sarasep CAR-Ca) was used with RI detection.

2.4.3 Manganese- and copper ion determination

Manganese- and copper ion concentrations in the growth media were determined by inductively coupled plasma quadrupole mass spectrometry (ICP-QMS,Thermo Fischer Scientific, Bremen, Germany). Hexapole Collison Cell Technology (CCT) was used. The reaction/collision gas was consisted 7% hydrogen 93% helium, the applied flow rate was 6 mL min^{-1}. The control of the instrument was performed by PlasmaLab software (ver. 2.5.10.319, Thermo Fischer Scientific). The calibration curves were always made freshly from mono elemental manganese reference solutions (1000 mg L^{-1} Mn (II), Scharlab S. L., Spain). Recovery was in the range of 95-100 %. The samples for Mn (II) were analysed at m/z 55, rhodium internal standard (20 µg L^{-1}) was used, and measured at m/z 103.

2.4.4 Dry cell weight determination

Mycelial dry cell weight (DCW) was determined from 5 mL culture aliquots. The biomass was harvested on a pre-weighted glass wool filter and washed with cold tap water, after which the filter was dried at 70°C until constant weight.

2.4.5 Investigation of the fungal morphology

Fungal morphology was investigated by means of an Axio-Vision AC quantitative image analyser system. To increase contrast and

visibility, lactophenol cotton blue (Fluka Chemie, Buch, Switzerland) was added to the samples in a final concentration of 10%. Stained samples were analysed under a Zeiss AxioImager phase-contrast microscope, equipped with AxioCam MRc5 camera. Average cell- and average pellet diameters (also referred to as micro- and macro-morphology, respectively) were assessed with the AxioVision AC image analyser system processing at least 50 cells or 10 pellets for each liquid culture sample studied.

2.5. Reproducibility

All presented data are the means of three to five independent experiments (biological replicates: starting with liquid cultures using different spore inocula). Data were analyzed and visualized with Sigmaplot software (Jandel Scientific), and for all datasets standard deviations were determined. Quantitative data ($n \geq 3$) were compared using ANOVA (Analysis of Variance) with Holm-Sidak Test for pairwise comparisons. The criterion for significance was $p < 0.05$ in all cases.

3. New scientific results

- Our results confirmed the need for Mn^{2+} ion deficiency of *A. terreus* NRRL 1960 D-xylose based fermentations as well. The findings support the interpretation that the effect of Mn^{2+} ions is independent of the carbon source. Without the manipulation of other media components, the manganese ion limitation is a key requisite to maximize itaconic acid yield.
- In contrast to the findings with D-glucose -where >100 g L^{-1} initial carbon source concentration required- this highest molar yield is already reached at 5% (w/v) D-xylose.
- Copper(II) ion tolerance of *Aspergillus terreus* NRRL 1960 depends on the carbon source. The germination of conidiospores tolerated much higher concentrations of copper - up to 3 g L^{-1} - for hexoses (D-glucose and D-fructose) in contrast with the pentoses (D-xylose and L-arabinose), where the tolerance reached 2 g L^{-1}.
- We demonstrated that, the concentrations of Cu^{2+} and Mn^{2+} influence the itaconic acid yield in a carbon source dependent manner. On hexose carbon sources, essentially the same itaconic acid yields could be reached, if copper: manganese ratio is set about 1000:1. However, contrary to the situation on the two glycolytic hexoses, the inhibitory effect of manganese(II) ion sufficiency on itaconic acid production was not fully alleviated

by an excess of copper(II) ions in the case of the pentoses as the growth substrate.

- We showed that, by elevating the KH_2PO_4 concentration in the fermentation medium accelerates carbon source consumption and rises itaconic acid productivity. On the other hand gives massive instability to the system. The specific molar yield of itaconic acid was halved in the presence of minimal inhibitory concentration of manganese ions in the fermentation broth, when the KH_2PO_4 concentration was elevated (0.8 g L^{-1}).
- We quantitative demonstrated the correlation between the fungal morphology and itaconic acid production. The best producers shows swollen, highly branched and even yeast-like morphology, while the increment of the pellet- and hyphal diameters along with the appearance of filamentous morphology disrupts the itaconic acid production.

4. Summary

During my doctoral scholarship period, itaconic acid production of the filamentous fungus *Aspergillus terreus* was investigated by studying the interplay of different media components on various carbon sources. In the last decade, the research focus of this area fell on finding new alternative carbon sources, in order to reduce production cost. Hydrolyzed plant biomass can be one such alternative carbon source. Hence, D-xylose was thoroughly investigated as sole carbon source in our experiments, as D-xylose is one of the major component of plant hydrolysates. Understanding the behavior of cultures on D-xylose could help to create a model system for itaconic acid fermentations on plant biomass hydrolysates.

D-glucose and D-xylose based fermentations were ran simultaneously, in order to investigate two different biochemical catabolic routes (Embden-Meyerhof-Parnas vs. pentose-phosphate pathway). Regarding manganese(II) ion sensitivity, *A. terreus* reacted in the same way on both carbon sources. Increasing the Mn(II) concentration over 5 μg L^{-1} resulted in significantly decreased specific molar itaconic acid yield. Thus molar yields are only able to reach their maximum at limiting manganese(II) ion concentrations (~1.5 μg L^{-1}).

D-glucose based fermentations resulted in 80% or higher specific molar yields only if initial glucose concentration in the medium is higher than 100 g L^{-1}. By employing D-xylose as sole carbon source, the specific

molar yield has reached the plateau at 50 g L^{-1} initial xylose concentration ($Y_{p/s}$ =0.61). Additional increase in the initial xylose concentration did not elevate this yield. Since we failed to get close to the theoretical maximal yields on D-xylose, the possibility of byproduct formation was also investigated. However, we did not detect any byproduct in higher amounts in the fermentation broth. According to our hypothesis, the specific biomass yield is lower (i.e., formation of the same amount of biomass requires more carbon) on D-xylose compared to D-glucose. Thus, one possible way to enhance itaconic yield on xylose based fermentations is to enhance the specific biomass yield ($Y_{x/s}$).

Subsequently, we concluded that manganese(II) ions negatively influence itaconic acid production. To reduce this effect, variations of the growth medium components were analyzed. Increasing the phosphate concentration in the growth medium even strengthened the adverse effects of manganese(II) ions, though the phenomenon was less profound during manganese(II) limitation. In contrast, at 10 μg L^{-1} Mn(II) concentration increased phosphate levels resulted in a significant (~ 40%) drop of the molar yield on both carbon sources compared to the phosphate limiting conditions. We concluded that the phosphate content of the medium must be limiting in order to achieve high-yield itaconic acid fermentations.

We successfully antagonized manganese(II) ions by increasing the Cu(II) concentration in D-glucose and D-fructose containing media.

In case of D-glucose, molar yield was fully restored ($Y_{p/s}$ = 0.82), whereas 90% of the yield achieved under manganese limiting conditions ($Y_{p/s}$ = 0.72) was recovered on D-fructose, in the presence of 300 μg L^{-1} Mn(II). A Cu(II):Mn(II) ratio of 1000:1 (equivalent to 300-400 mg L^{-1} Cu^{2+}) was required in the growth media to achieve this. However, this approach was less successful on D-xylose as sole carbon source, as we could increase the molar yield only to 25% with 100 mg L^{-1} Cu(II). Using L-arabinose resulted in the worst itaconic acid yields, as we obtained only 32% conversion under manganese limitation, and only negligible amounts of itaconic acid was formed in the presence of 300 μg L^{-1} Mn(II). We also demonstrated that the resistance of *A. terreus* NRRL 1960 against copper toxicity is carbon source dependent, and displays much higher values in hexose-containing media than on pentoses. The reason behind the phenomenon could be the more limited ATP supply on pentose growing cultures. Filamentous fungi mainly keep their copper homeostasis by ATPase enzymes, which remove the excessive copper ions from the cytoplasm by consuming ATP . This mechanism may function less effectively on pentose carbon sources, than on hexoses.

High molar conversions of itaconic acid and fungal morphology showed strong correlation to each other. Globular, rounded, swollen cells were formed, with 8-10 μm hyphal diameters, in the case of manganese limitation (1.5 μg L^{-1} Mn^{2+}), while semi-filamentous morphology was observed at 5 μg L^{-1} Mn(II). In the presence of 10 μg L^{-1} Mn(II), or above this concentration, the cultures formed thin, long hyphae of around 2 μm

diameter. Increasing the KH_2PO_4 concentration from 0.1 g L^{-1} to 0.8 g L^{-1} did not influence hyphal diameters in general, furthermore, did not influence pellet diameters in the case of manganese limitation, whilst supported the formation of larger pellets (>450 μm) at the presence of 10 μg L^{-1} Mn(II). The molar yields always reflected morphology.

By using optimized growth media (phosphate and manganese limitation), small pellets of 100 μm (or even smaller) diameters formed at 3.3 mg L^{-1} initial Cu(II) concentration. These cultures usually perform >80% molar yields, while addition of 300 μg L^{-1} Mn(II) to the media resulted in the formation of large (>350 μm) pellets that give lower itaconic acid yields. The addition of excess copper ions to the fermentation broth keeps pellet diameters below 200 μm, with fully recovered molar yields.

In conclusion, during high-yield itaconic acid fermentations, small pellets (up to 200-300 μm diameter), and globular cells with wide hyphae were observed. In contrast, fermentations with low itaconic acid molar yields showed thin and long hyphae, with huge pellets. The adequate morphology and biomass concentration assist the development of a favorable rheology in the fermented broth , which might correlate with the (alternative, cyanide-resistant) respiration of the fungus. Lower viscosity in the culture broth increases transport rates in the medium. Oxygen and other nutrients can easier reach the center of the small pellets, thereby supporting the overflow metabolism towards itaconic

acid production. Hence, itaconic acid production by *A. terreus* shows strong dependence on the fungal morphology, and the energy level (ATP availability) of the cells.

Publications

Lectures on conferences:

Kolláth, IS: Cu(II)- és Mn(II)-ionok eltérő koncentrációinak hatása az *Aspergillus terreus* fonalas gomba itakonsav termelésére és morfológiájára (New National Excellence Programme Conference, Debrecen, Hungary, 2019)

Kolláth, IS: Cu(II)-ionok hatása az *Aspergillus terreus* fonalas gomba itakonsav termelésére (Magyar Mikrobiológiai Társaság 2018. évi Nagygyűlése, Eger, Hungary, 2018)

Kolláth, IS ; Fekete, E ; Sándor, E ; Soós, Á ; Kovács, B ; Karaffa, L: Effect of copper (II) ions on itaconic acid production by *Aspergillus terreus* (7th Interdisciplinary Doctoral Conference, Pécs, Hungary, 2018)

Scientific posters presented in conferences:

Kolláth, IS ; Fekete, E ; Karaffa, L: Comparative performance of *Aspergillus terreus* itaconic acid fermentations on D-xylose and xylitol (15th European Conference of Fungal Genetics, Rome, Italy, 2020)

Kolláth, IS ; Fekete, E ; Karaffa, L.: Az *Aspergillus terreus* gomba itakonsav termelésének vizsgálata D-xilóz, illetve xilitol szénforrásokon (Biotechnológia a Debreceni Egyetemen-2019 tudományos szimpózium, Debrecen, Hungary, 2019)

Kolláth, IS ; Fekete, E ; Karaffa, L.: Itaconic acid production by *Aspergillus terreus* from D-xylose and xylitol (18th International Congress of the Hungarian Society for Microbiology, Budapest, Hungary, 2019)

Karaffa, L ; Molnár, ÁP ; Kolláth, IS ; Kovács, B. ; Soós, Á. ; Kubicek, CP ; Fekete, E: *Aspergillus terreus* itaconic acid fermentation technology reflects the physiological requirements of overflow metabolism (11th International Mycological Congress, San Juan, Puerto Rico, 2018)

Kolláth, IS ; Molnár, ÁP ; Fekete, E ; Karaffa, L: Itaconic acid production from D-xylose by *Aspergillus terreus* (14th European Conference on Fungal Genetics, Haifa, Israel, 2018).

Kolláth, IS ; Molnár, ÁP ; Fekete, E ; Sándor, E ; Soós, Á ; Kovács, B ; Kubicek, CP ; Karaffa, L: Production of itaconic acid from D-xylose by *Aspergillus terreus*: (5th Central European Forum for Microbiology [CEFORM], Keszthely, Hungary, 2017).

Kolláth, IS ; Molnár, ÁP ; Fekete, E ; Sándor, E ; Soós, Á; Kovács, B ; Kubicek, CP ; Karaffa, L: Production of itaconic acid from D-xylose by *Aspergillus terreus* (2nd Symposium on Plant Biomass Conversion by Fungi, Utrecht, Netherlands, 2017).

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/536/2021.PL
Subject: PhD Publication List

Candidate: István Sándor Kolláth
Doctoral School: Doctoral School of Chemistry
MTMT ID: 10060294

List of publications related to the dissertation

Foreign language scientific articles in international journals (2)

1. Sándor, E., **Kolláth, I. S.**, Fekete, E., Bíró, V., Flipphi, M., Kovács, B., Kubicek, C. P., Karaffa, L.: Carbon-source dependent interplay of copper and manganese ions modulates the morphology and itaconic acid production in Aspergillus terreus.
Front. Microbiol. Epub, 1-14, 2021. EISSN: 1664-302X.
DOI: http://dx.doi.org/10.3389/fmicb.2021.680420
IF: 5.64 (2020)

2. **Kolláth, I. S.**, Molnár, Á. P., Soós, Á., Fekete, E., Sándor, E., Kovács, B., Kubicek, C. P., Karaffa, L.: Manganese Deficiency Is Required for High Itaconic Acid Production From D-Xylose in Aspergillus terreus.
Front. Microbiol. 10, 1-10, 2019. EISSN: 1664-302X.
DOI: http://dx.doi.org/10.3389/fmicb.2019.01589
IF: 4.235

List of other publications

Foreign language scientific articles in international journals (1)

3. Molnár, Á. P., Németh, Z., **Kolláth, I. S.**, Fekete, E., Flipphi, M., Ág, N., Soós, Á., Kovács, B., Sándor, E., Kubicek, C. P., Karaffa, L.: High oxygen tension increases itaconic acid accumulation, glucose consumption, and the expression and activity of alternative oxidase in Aspergillus terreus.
Appl. Microbiol. Biotechnol. 102 (20), 8799-8808, 2018. ISSN: 0175-7598.
DOI: http://dx.doi.org/10.1007/s00253-018-9325-6
IF: 3.67

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Foreign language abstracts (4)

4. Karaffa, L., Molnár, Á. P., **Kolláth, I. S.**, Kovács, B., Soós, Á., Kubicek, C. P., Fekete, E.: Aspergillus terreus itaconic acid fermentation technology reflects the physiological requirements of overflow metabolism.
   In: 11th International Mycological Congress: Mycological Discoveries for a Better World : Abstract Book, Ana G. Méndez University, San Juan, Puerto Rico, 355-356, 2018.

5. **Kolláth, I. S.**, Fekete, E., Sándor, E., Soós, Á., Kovács, B., Karaffa, L.: Effect of copper (II) ions on itaconic acid prodoction by Aspergilius terreus.
   In: VII. Interdiszciplináris Doktorandusz Konferencia 2018 absztraktkötet. Szerk.: Bódog Ferenc, Pécsi Tudományegyetem Doktorandusz Önkormányzat, Pécs, 74, 2018. ISBN: 9789634292104

6. **Kolláth, I. S.**, Molnár, Á. P., Fekete, E., Sándor, E., Soós, Á., Kovács, B., Kubicek, C. P., Karaffa, L.: Production of itaconic acid from D-xylose by Aspergillus terreus. Utánközlés párhuzamos közlés,
   In: 2nd Symposium on Plant Biomass Conversation by Fungi, Westerdijk Fungal Biodiversity Institute, Utrecht, Hollandia, 25, 2017.

7. **Kolláth, I. S.**, Molnár, Á. P., Fekete, E., Sándor, E., Soós, Á., Kovács, B., Kubicek, C. P., Karaffa, L.: Production of itaconic acid from D-xylose by Aspergillus terreus.
   In: Abstracts of the 5th Central European Forum for Microbiology. Ed.: K. Márialigeti, O. Dobay, Akadémiai Kiadó, Budapest, 136, 2017, (Acta Microbiologica et Immunologica Hungarica, ISSN 1217-8950 ; 64. (Suppl))

Total IF of journals (all publications): 13,545
Total IF of journals (publications related to the dissertation): 9,875

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

21 December, 2021

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *