UNIVERSITY OF PANNONIA

CHEMICAL ENGINEERING AND MATERIAL SCIENCES DOCTORAL SCHOOL

APPLICATION OF *ASPERGILLUS TERREUS* STRAIN FOR THE PRODUCTION OF ITACONIC ACID

PH.D. THESES

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INTRODUCTION

The chemical industry is a major contributor to environmental pollution, but it provides essential raw materials for all other technologies. Current biotechnological alternatives to large-scale chemical processes are not economically competitive as long as the possibility for a petroleum-based industry is given. However, the key to future biotechnology may lie in the development of continuous fermentation processes and minimising water-, energy and feedstock consumption.

The integration of membrane separation processes offers a promising avenue for improvement. In case of organic acid production, bipolar electrodialysis is of particular importance, since chemical-free recovery of the acidic product can be achieved from aqueous solution.

In the integrated fermentation-bipolar electrodialysis concept, in a continuous fermentation with nutrients remaining in the effluent alongside the product, separation of the product is achieved by membrane operation and the residual solution is recycled after appropriate treatment in the feed liquid stream.

In light of this, my aim was to establish the production of itaconic acid by continuous fermentation. Itaconic acid is a small molecule containing double bonds, may be a precursor for complex chemical syntheses, and it can be produced by the fungus *Aspergillus terreus* from simple sugars in technologically relevant concentrations.

Three main areas of the fermentation process were addressed: (1) the influence of the inoculum on the process; (2) the feasibility of continuous fermentation with relevant product concentration; and (3) the effect of light as an environmental parameter on the development of *Aspergillus terreus* and the fermentation process.

EXPERIMENTAL

In the experiments, a variety of culturing and fermentation techniques were applied in an arrangement to measure the effect of the parameters under investigation.

In comparative shake-flask fermentations of two effective itaconic acid producing *Aspergillus terreus ssp.*, it was investigated how the inoculation spore concentration influences the process.

To establish the basis for continuous fermentation, I performed semi-continuous fermentation experiments, in which, the loading strategies of glucose, as carbon source were analysed. For this purpose, Sartorius Biostat bench-top fermenters were used with built-in measuring electrodes and data logger.

Continuous fermentation was carried out in a Lambda bench-top fermenter, at low dilution rates to maximise the itaconic acid concentration in the effluent. After recovery of the itaconic acid by electrodialysis from the effluent stream, the liquid was recycled back to the continuous fermentation, thus achieving recirculation of the residual glucose. This was the first occasion that the feasibility of the integrated fermentation-electrodialysis system was investigated for itaconic acid production.

Solid-state and shake-flask fermentations were carried out with the aim of characterizing the effect of illumination on the itaconic acid-producing *Aspergillus terreus*. During my experiments the issue of itaconic acid-induced product inhibition was also addressed, which may be a limitation of continuous fermentation.

NOVEL SCIENTIFIC ACHIEVEMENTS

In my Ph.D. work, the following scientific results have been achieved:

1. ***Studying the impact of inoculation on the fermentation process, it was found that:***

- **in the case of shaken flask fermentation with *Aspergillus terreus* DSM826, when the inoculum is a suspension of conidia, the initial spore concentration has a significant effect on the dry mycelial weight concentration (DMW) formed, while the final itaconic acid titer does not change.**

  At low inoculum spore concentrations - 10^4 - 10^5 spores/ml, 5 g/l DMW was formed until glucose depletion, while a value above 8 g/l DMW was measured for the range 10^6 - 10^7 spores/ml.

- **an optimal range of values for the inoculum spore number is valid for the fermentation of the yeast *Aspergillus terreus* DSM826.**

At low spore concentrations of $1 \cdot 10^6$ spores/ml, the process is slower but the yield of itaconic acid is higher than in the range $5\text{-}11 \cdot 10^6$ spores/ml. At $1.1 \cdot 10^7$ spores/ml, the process is also slower than at the initial spore count of $5\text{-}8 \cdot 10^6$ spores/ml. At $1.1 \cdot 10^7$ spores/ml, spore germination is inhibited and yields are at a minimum under the experimental conditions.

2. ***Based on the semi-continuous and continuous fermentation experiments, it was found that:***

- **semi-continuous fermentation with *Aspergillus terreus* DSM826, with 24 h intervention cycles and 13.4% volume changes, results in periodic changes in productivity and yield.**

  Productivity minima ranges from 0.05-0.17 g/l/h and maxima from 0.23-0.27 g/l/h. Yield minima ranges from 0.07-0.25 g/g and maxima from 0.28-0.50 g/g [1].

- **in semi-continuous fermentation *Aspergillus terreus* DSM826 (24 h intervention cycles and 13.4% volume changes), restoring the 120 g/l initial glucose content in the fermentation broth during interventions leads to a doubling of mycelial mass compared to a constant 100 g/l glucose feed.**

  The dry mycelial weight concentration increased from 10 g/l to 20 g/l, while no significant increase in yield or productivity occurred [1].

- **continuous itaconic acid fermentation, that significantly exceeds the achievable product concentration in relevant literature value, is feasible applying *Aspergillus terreus* DSM826 free mycelium as producing cell mass.**

  At a low dilution rate of 0.007 1/h, an average of 29 g/l of itaconic acid was produced in a 1.7 L fermentor [2].

3. ***Studying the impact of illumination with white light on the development of Aspergillus terreus DSM826, it was found that:***

- **constant illumination with 70 lux white light can increase the number of conidia per unit area of complex potato glucose agar (PGA) medium by a factor of 1.8 compared to dark incubated cultures of *Aspergillus terreus* DSM826 strain [8].**
- **speaking of conidia based inoculation, there is no significant difference in the productivity of itaconic acid in batch fermentations, depending on whether inoculum produced under constant light or dark conditions is used, provided that they have the same spore concentration.**

- **if 30 g/l of itaconic acid is added to the fermentation medium prior to inoculation, the mycelial mass developing from conidia under constant illumination produces significantly less by-products than cultures incubated in the dark. The production of coloured metabolites is also inhibited [8].**
- **if 30 g/l of itaconic acid is added to the fermentation medium before inoculation, then under continuous 70 lux illumination, significantly more itaconic acid is produced and more glucose is consumed during a one-week batch process compared to cultures incubated in dark, while the yield and mycelial mass produced are not significantly different.**

  Thus, at an initial itaconic acid content of 30 g/l, constant illumination stimulates itaconic acid production but has no effect on metabolic stoichiometry.

LIST OF PUBLICATIONS

I. **Hülber-Beyer, É.**, Bélafi-Bakó, K., Rózsenberszki, T., Komáromy, P., & Nemestóthy, N. (2023) Evaluating the potential of semi-continuous itaconic acid fermentation by *Aspergillus terreus*: operational profile and experiences. *World Journal of Microbiology and Biotechnology*, *39*, 346. https://doi.org/10.1007/s11274-023-03797-9

II. **Hülber-Beyer, É.**, Bélafi-Bakó, K., & Nemestóthy, N. (2023). Case study of continuous itaconic acid fermentation by *Aspergillus terreus* in a bench-scale bioreactor. *Hungarian Journal of Industry and Chemistry*, *51*(2), 57–63. https://doi.org/10.33927/hjic-2023-19

III. **Hülber-Beyer, É.**, Bélafi-Bakó, K., & Nemestóthy, N. (2021). Low-waste fermentation-derived organic acid production by bipolar membrane electrodialysis—an overview. *Chemical Papers*, *75*(10). https://doi.org/10.1007/s11696-021-01720-w

IV. **Hülber-Beyer, É.**, Bélafi-Bakó, K., & Nemestóthy, N. (2020). Az eredő folyadékoldali térfogati oxigénabszorpciós együttható (K_La) meghatározása itakonsav fermentációnál. (Determination of the volumetric oxygen mass transfer coefficient (K_La) for the implementation of itaconic acid fermentation) *Membrántechnika és Ipari Biotechnológia, 3*(XI.), 26-37. ISSN: 2061-6392

V. Komáromy, P., Bélafi-Bakó, K., **Hülber-Beyer, É.**, & Nemestóthy, N. (2020). Enhancement of Oxygen Transfer through Membranes in Bioprocesses. *Hungarian Journal of Industry and Chemistry*, *48*(2), 5–8. https://doi.org/10.33927/hjic-2020-21

VI. Rózsenberszki, T., Komáromy, P., **Hülber-Beyer, É.**, Bakonyi, P., Nemestóthy, N. & Bélafi-Bakó, K. (2021). Demonstration of bipolar membrane electrodialysis technique for itaconic acid recovery from real fermentation effluent of *Aspergillus terreus*. *Chemical Engineering Research and Design*, *175*(2021), 348–357. https://doi.org/10.1016/j.cherd.2021.09.022

VII. Rózsenberszki, T., Komáromy, P., **Hülber-Beyer, É.**, Pesti, A., Koók, L., Bakonyi, P., Bélafi-Bakó, K. & Nemestóthy, N. (2023). Bipolar membrane electrodialysis integration into the biotechnological production of itaconic acid: A proof-of-concept study. *Chemical Engineering Research and Design*, *190*, 187-197. https://doi.org/10.1016/j.cherd.2022.12.023

VIII. **Hülber-Beyer, É.**, Tóth, G. & Bélafi-Bakó, K. (2022). Bioszorpció *Aspergillus terreus* fonalas gomba biomasszával. (Biosorption with the biomass of the filamentous fungus *Aspergillus terreus*) *Membrántechnika és Ipari Biotechnológia, 4*(XIII.), 38-43. ISSN: 2061-6392

Poster and Oral Presentation

Hülber-Beyer, É., Nemestóthy, N. & Bélafi-Bakó, K. (2021) Production of itaconic acid by continuous fermentation – bipolar membrane electrodialysis integrated system. *Interdisciplinary Doctoral Conference*, 2021. november 12-13. Pécs, Magyarország. (poster)

Hülber-Beyer, É., Bélafi-Bakó, K. & Nemestóthy, N. (2022) Influence of light on the itaconic acid producer *Aspergillus terreus. 48th International conference of SSCHE, 2022. május 23-26.* Szlovákia, Tatranske Mateliare. (presentation)