Fermentation, Downstream Processing and Isolation of Biologically Active Terpenoids From Cultures of *Cyathus* and *Hericium* spp. (Basidiomycota)

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CLIB 2021 CLUSTER INDUSTRIELLE BIOTECHNOLOGIE

Projekt 2: Optimierung der Zugänglichkeit mikrobieller Sekundärmetabolite

Bundesministerium für Bildung und Forschung

Workshop „Rote und Weiße Biotechnologie: Herstellung von Substanzen mittels Fermentationsverfahren, deren Aufarbeitung und Reinigung“
22 April.2010 - Materials Valley, Hanau, Germany

Agenda

- **Rationale, objectives, and working plan of the project**
- ***Cyathus* and *Hericium* as model organisms**
- **Optimisation of fermentation**
- **Optimisation of downstream processing & CPC separation technology**
- **Outlook and Discussion**

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- Outlook and Discussion

Rationale & objectives

- **Increase accessibility of natural products through development of new methods of downstream processing & innovative chromatography procedures**

- **Development of heuristic-based processes to synthesise process concepts based on physical property data and few scouting experiments**

- **<u>Technical goal</u>: Advancement & scale-up of CPC (Centrifugal Partition Chromatography) as alternative to conventional liquid chromatography**

➢ **Accelerate development of biologically active secondary metabolites, thereby increasing their market chances & intrinsic value significantly**

See for project summary:
http://www.clib2021.com/fileadmin/templates/pdf/090511_EN_IntermedDiscovery.pdf

Overview of working program

WP	Task
1	✓ Screening of producer strains (small scale fermentation)
2	➢ Scale-Up of fermentation (30 litre scale)
3	➢ Preparation & characterisation of crude extracts
4	➢ Characterisation of major metabolites
5	➢ Screening of downstream processing methods
6	➢ Optimisation of Batch-CPC methodology
7	o Evaluaton of alternative process concepts
8	o Economical and ecological assessment
9	o Design of a manual for secondary metabolites
10	o Patents, dissemination, etc.

Project start in Feb. 2009; scheduled for 36 months

✓ done ➢ ongoing o planned for 2010/2011

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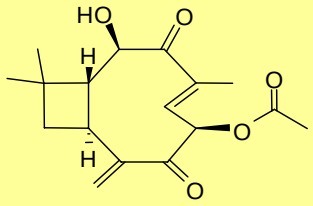
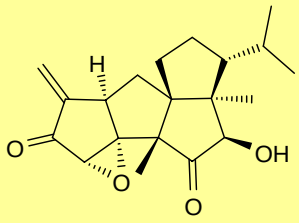
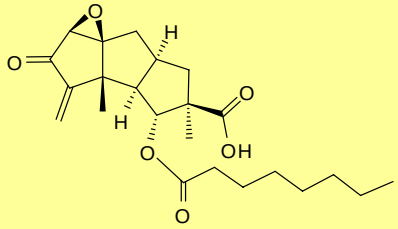
Terpenoids from basidiomycetes (1)



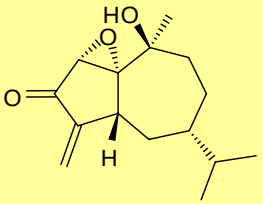
Naematolon (*Hypholoma*)



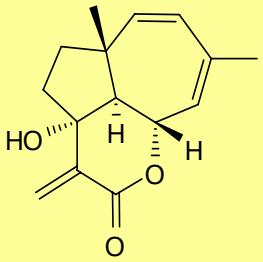
Crinipellin A (*Crinipellis*)



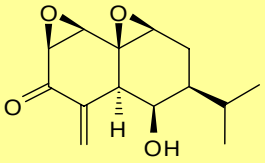
Phellodonic acid (*Phellodon*)



Fimicolon *Panaeolus*)

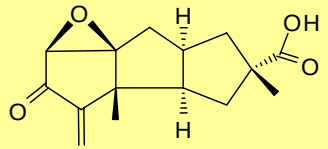


Fulvoferruginin (*Marasmius*)



Panellon (*Panellus*)

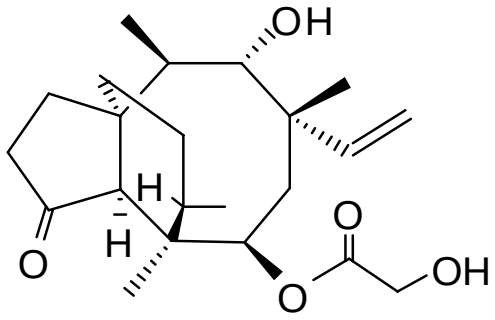
Several hundreds of unique biologically active terpenoids are known from cultures of Basidiomycetes



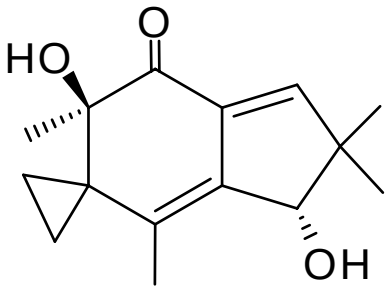
Complicatic acid (*Stereum*)

Source of fungal images: *http://www.mycokey.org*

Terpenoids from basidiomycetes (2)



Pleuromutilin
Clitopilus spp.
("*Pleurotus mutilis*")
Antibacterial antibiotic
(semi-synthetic derivatives => marketed drugs)



Illudin-M
Omphalotus olearius
("*Clitocybe illudens*")
Anticancer agent
(semi-synthetic derivatives in development)

Source of fungal images: *http://www.mycokey.org*

Challenges in exploitation of fungal terpenoids

- **Low intrinsic yields** of desired compounds
- Slow-growing producer organisms, showing shear sensitivity and **lack of production** in stirring tanks upon scale-up
- Production of mixtures of several congeners afford **extensive preparative** isolation work
- **Instability** during extraction & chromatography often observed
- **Heterologous expression systems are not (yet) available**

Potential future applications of fungal terpenoids

Numerous previously discovered fungal metabolites with interesting biological activities were abandoned, due to **lack of sustainable access**

- Especially compounds from **edible species** will have good chances in the future to be developed as food ingredients and flavor components (high market demand for innovative products)

- Recent progress in **molecular genetics and genomics** offers unprecedented options for rational design of novel natural drugs, pesticides and other products for the Life Science industries

However, the availability of a concise strategy for production & downstream processing will remain indispensable for success !

Bird's Nest, Hedgehog, and Tooth Fungi

Cyathus striatus

C. olla

Hericium coralloides

H. erinaceus

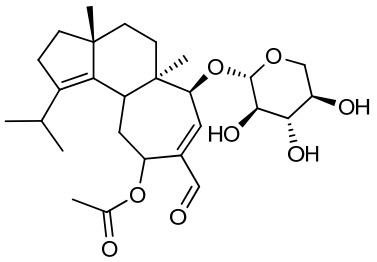
H. cirrhatum

Source of fungal images: http://www.mycokey.org

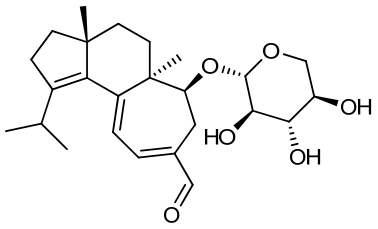
Producers of Cyathane Diterpenoids

Hericium coralloides

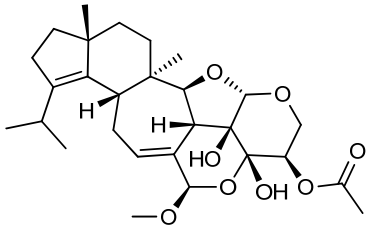
Cyathus striatus



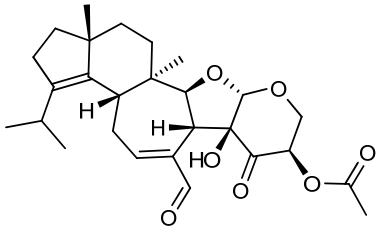
Herical



Erinacin A



Striatin A



Striatal A

Model Terpenoids

Antibiotic, anticancer, and other interesting biological activities

(several hundreds of further bioactive metabolites and other compounds of potential prectical relevance are known from plants and fungi)

Source of fungal images: http://www.mycokey.org

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Habit of mycelial cultures

Cyathus striatus

Hericium erinaceus

Most cultures studied were isolated in-house or obtained via IMD's world-wide collaboration network

Strains used for screening

Strain No.	Genus	Speciast	Origin
FU70494	*Crucibulum*	*laeve*	**MS**
FU70638	*Cyathus*	*africanus*	**P**
FU02817	*Cyathus*	*olla*	**MS**
FU04395	*Cyathus*	*olla*	**MS**
FU40495	*Cyathus*	*olla*	**C**
FU70655	*Cyathus*	*olla*	**P**
FU04515	*Cyathus*	*sp.*	**MS**
FU70640	*Cyathus*	*sterocoreus*	**P**
FU02747	*Cyathus*	*striatus*	**P**
FU04405	*Cyathus*	*striatus*	**MS**
FU40496	*Cyathus*	*striatus*	**C**
FU70492	*Cyathus*	*striatus*	**MS**
FU70493	*Cyathus*	*striatus*	**MS**
FU70639	*Cyathus*	*striatus*	**P**
FU70641	*Cyathus*	*striatus*	**P**
FU70654	*Cyathus*	*striatus*	**P**

Strain No.	Genus	Speciast	Origin
FU71745	*Hericium*	*alpestre*	**P**
FU71746	*Hericium*	*cirrhatum*	**P**
FU40484	*Hericium*	*coralloides*	**C**
FU46483	*Hericium*	*coralloides*	**C**
FU70125	*Hericium*	*coralloides*	**C**
FU71982	*Hericium*	*coralloides*	**MS**
FU71747	*Hericium*	*coralloides*	**P**
FU71748	*Hericium*	*coralloides*	**P**
FU71983	*Hericium*	*erinaceus*	**MS**
FU04826	*Hericium*	*erinaceus*	**MS**
FU64511	*Hericium*	*erinaceus*	**MS**
FU64523	*Hericium*	*erinaceus*	**MS**
FU70034	*Hericium*	*erinaceus*	**C**

Legends: **P**: Public collection; **C**: IMD collaboration network; **MS**: personal herbarium/culture collection of M. Stadler

Over 30 different cultures of *Cyathus*, *Hericium* and related genera from different geographic regions were so far screened for diterpene production

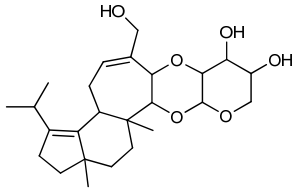
Fermentation techniques

Preliminary experiments in shake flasks

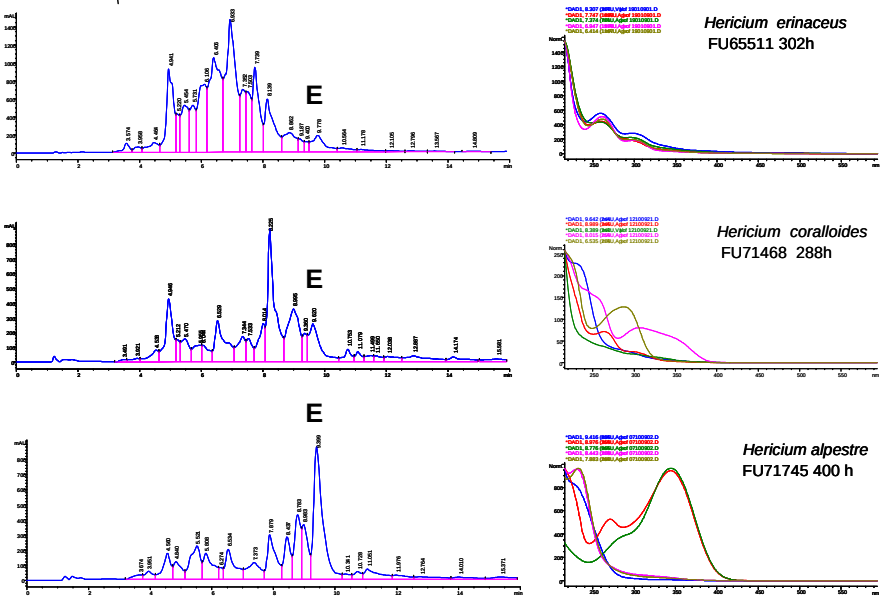
=> Scale up to 10 and 30 litre scale (stirring fermentors)

= most crucial step in our experience

Preliminary screening & HPLC profiling



Erinacin C (**E**)



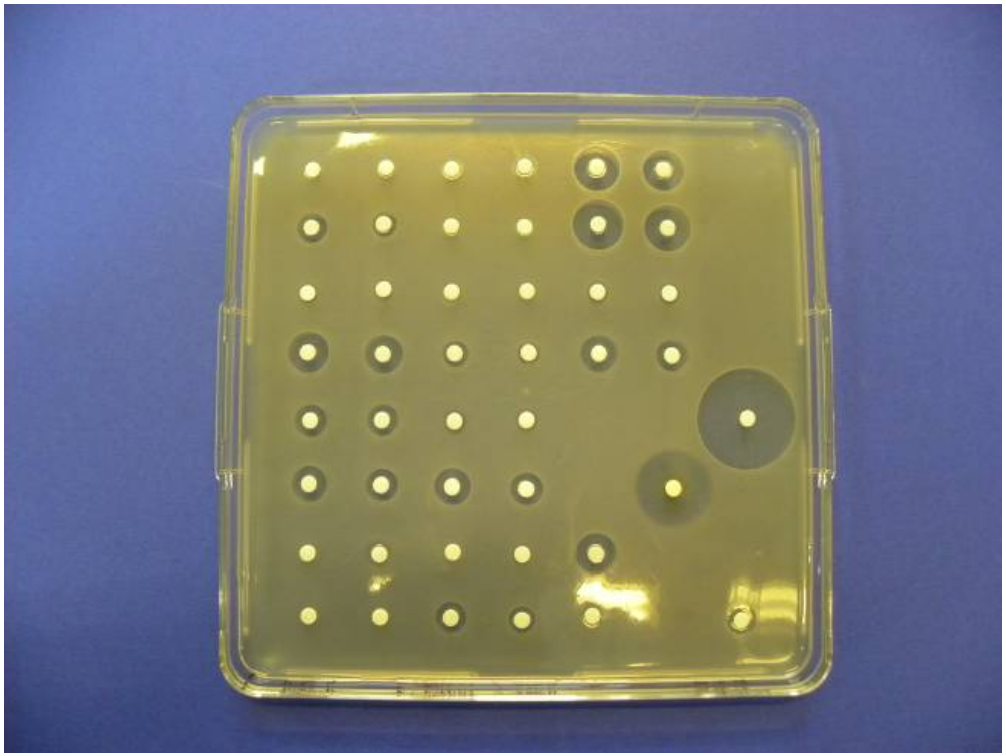
30 strains of *Cyathus* and *Hericium* spp. were studied for production of cyathane diterpenoids by

a) HPLC profiling (DAD; ESI-MS)

b) monitoring of antibiotic activities during fermentation in different culture media

- Selection of best producer organism; optimisation of culture media, fermentation time etc. for scale up & intensified studies
- Detection of potentially new congeners for preparative isolation

Concurrent monitoring of antibiotic effects



Conventional, well established methods (e.g. agar diffusion assay)
using sensitive test organisms allow for concise selection of producer strains, as well as for elaboration of optimal fermentation procedures

HPLC-MS dereplication

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Chimia 61 (2007) 332–338

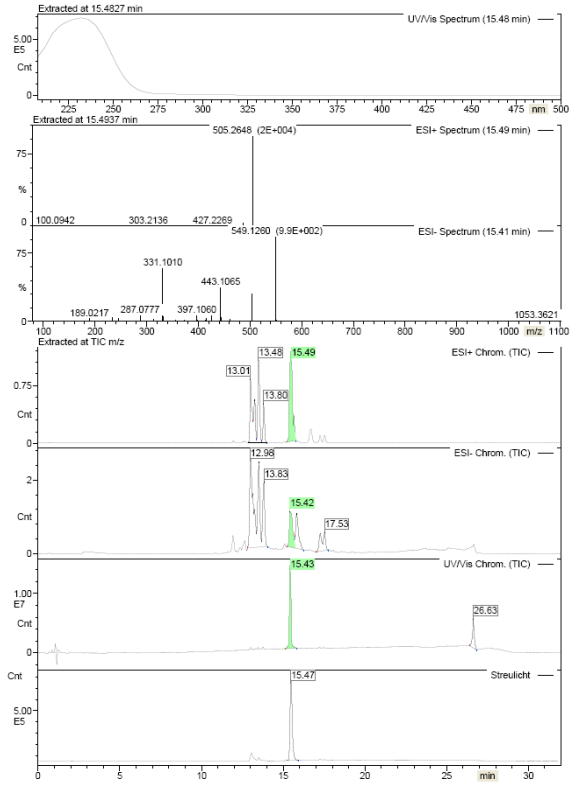
ISSN 0009–4293

Accelerated Dereplication of Natural Products, Supported by Reference Libraries

Jens Bitzer[a], Bärbel Köpcke[a], Marc Stadler[a], Veronika Hellwig[b], Yu-Ming Ju[c], Stephan Seip[d], and Thomas Henkel*[a]

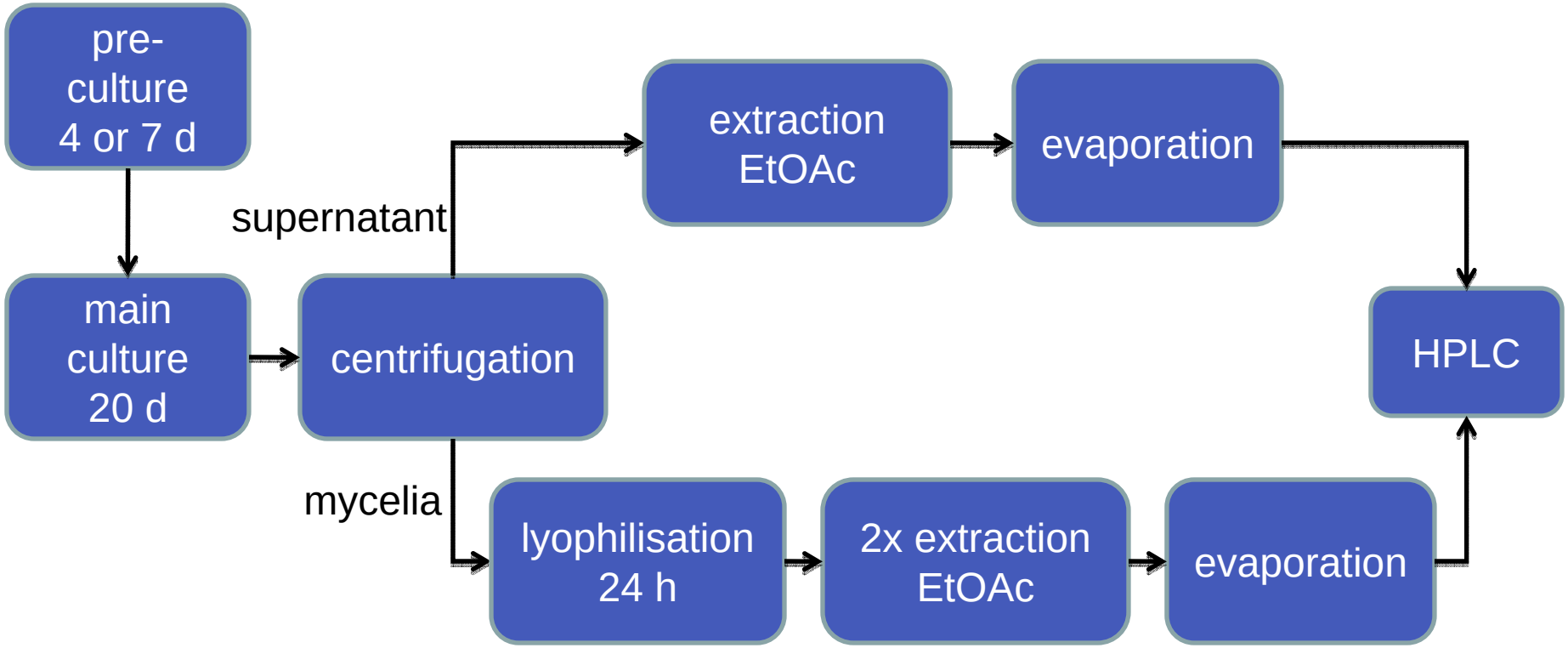
Abstract: Natural products are an indispensable source for drug discovery. The major challenge for exploiting this evolutionary optimized pool of potential lead structures is the fast and reliable recognition of known compounds, *i.e.* dereplication. This task is essential for the discovery process in high-throughput screening scenarios, since it allows the focus to be placed on novel chemical structures at an early stage. Furthermore, information on identified compounds will help to rationalize observed bioactivities. This article describes an effective, library-supported strategy for the dereplication of crude extracts and pre-fractionated samples, using an HPLC-based multidetector platform and NMR techniques, respectively.

Keywords: Dereplication · Multidetector HPLC · Natural products · NMR · Secondary metabolites · Tandem mass spectrometry



Proprietary technology of InterMed Discovery GmbH, based on the world-wide largest library of pure natural products, is available for characterisation of crude extracts

Workup scheme for analysis of striatal production



Tian Shen, Diploma thesis, JLU Gießen (2009)

Methodology

Culture Conditions:

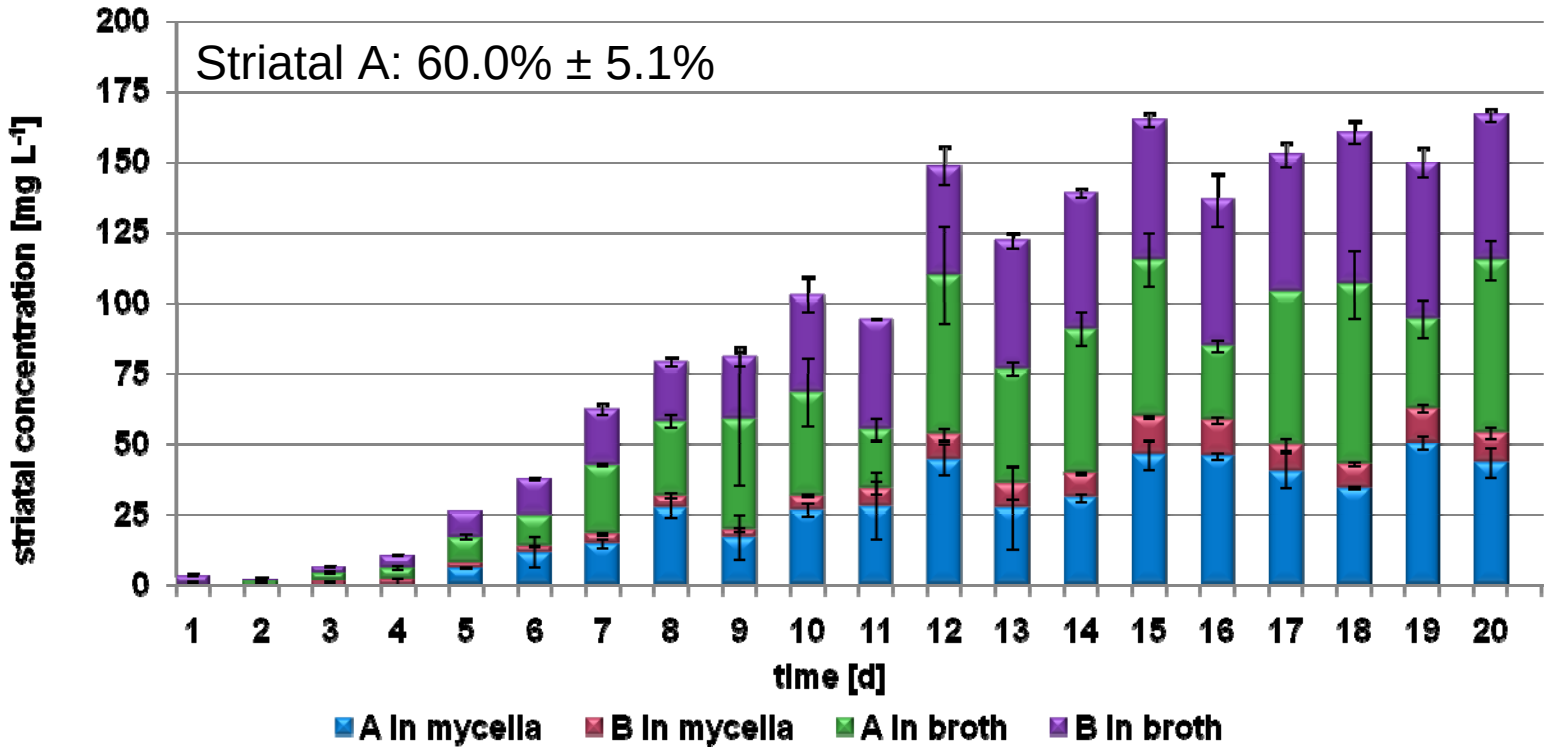
- media screening: e.g., SNL, yeast malt glucose (YM), soy peptone (SP)
- shake cultures (24 °C, 150 rpm)
- cultivation dark or irradiated with UV-A light (300 – 400 nm),
  10 h UV-A irradiation, 14 h darkness rhythm

Evaluation of Growth/Production Parameters:

- Biomass: wet & dry weight
- pH Value
- Free glucose concentration of culture broth
- Striatal concentration in culture supernatant and in mycelia
  estimated by HPLC-DAD, using standards of striatals A & B

Tian Shen, Diploma thesis, JLU Gießen (2009)

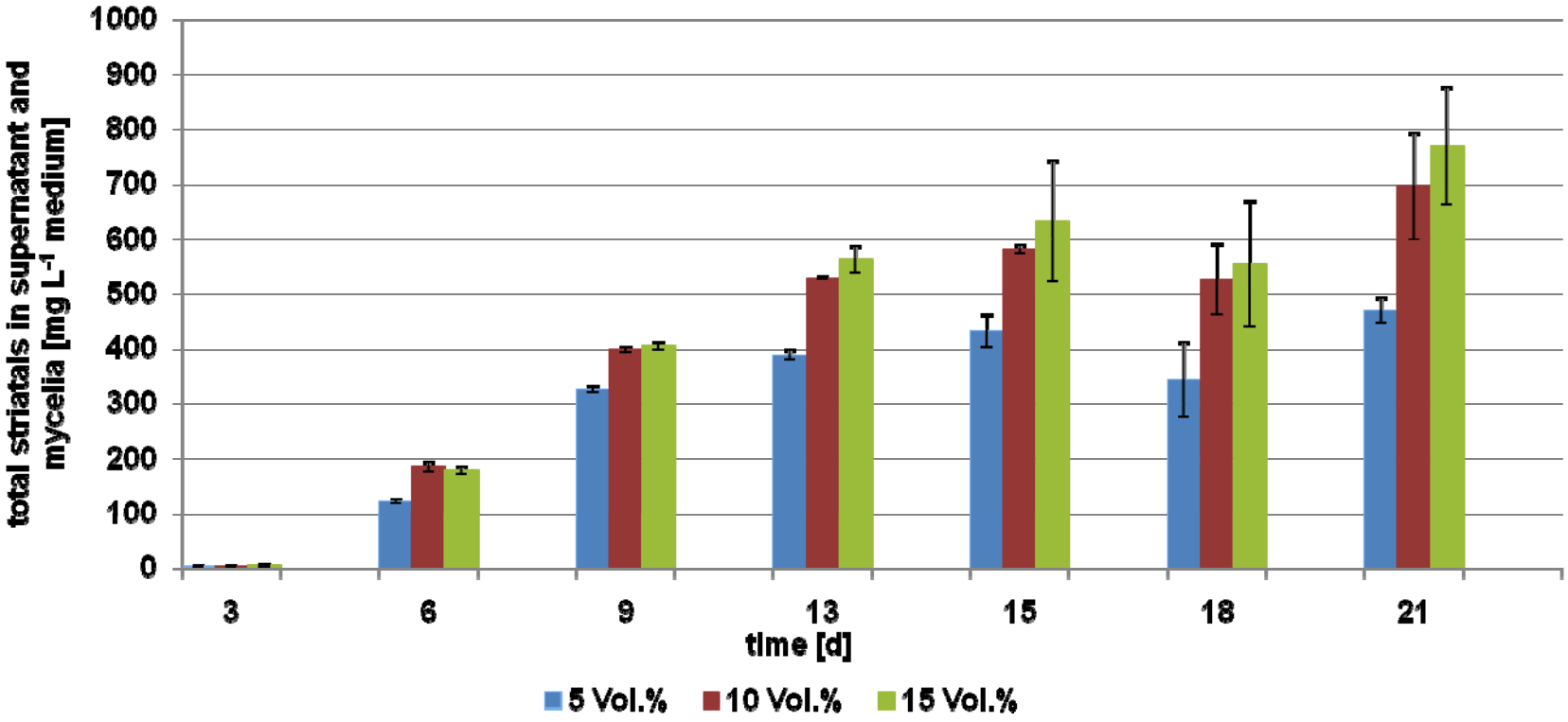
Ratio of Striatal Production



Striatals A and B are always produced concurrently,
and located in both the culture broth and mycelia of *C. striatus*

Tian Shen, Diploma thesis, JLU Gießen (2009)

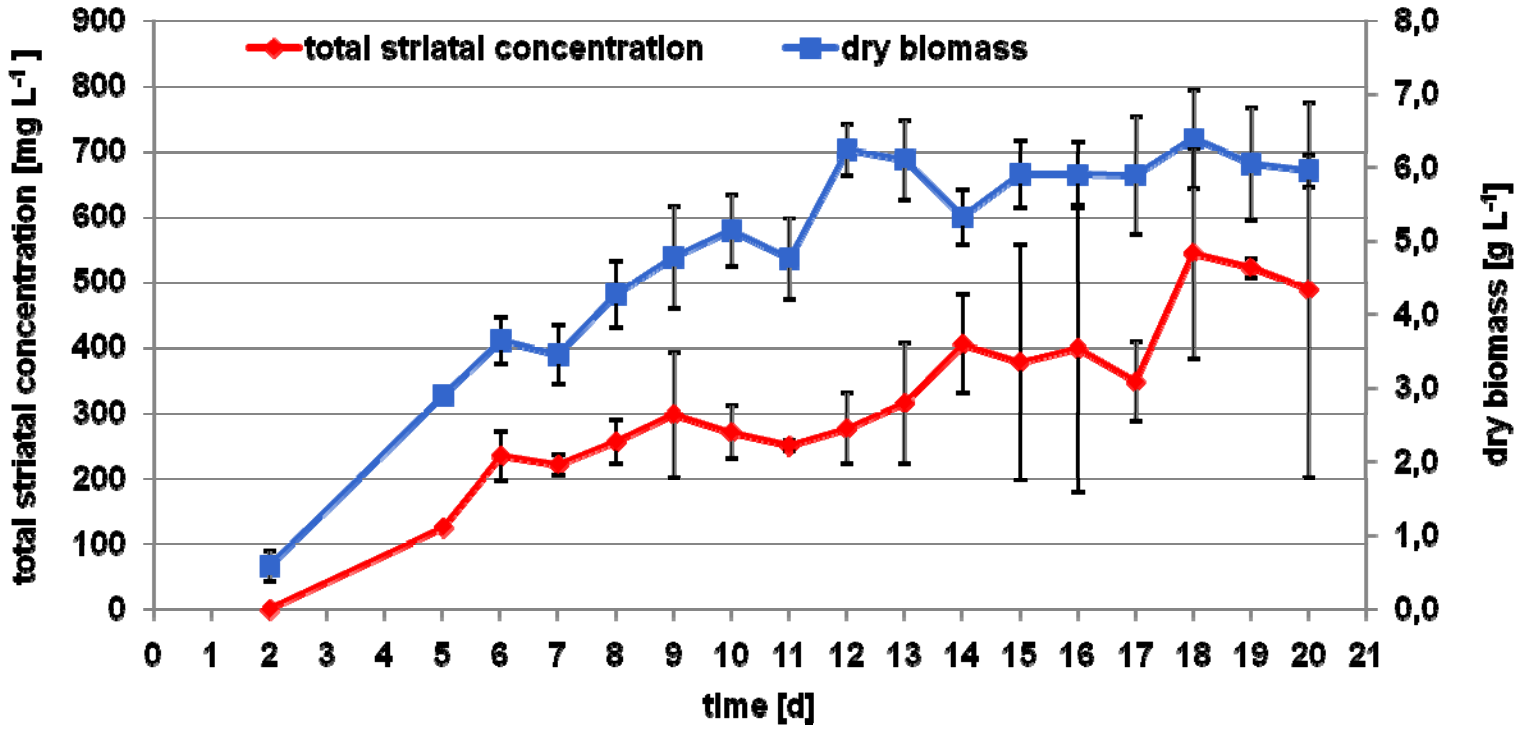
Time Course of Striatal Production



Inoculum size has some effects on final yields of striatals
However, higher inoculum rates do not necessarily accelerate production

Tian Shen, Diploma thesis, JLU Gießen (2009)

Correlation of Biomass & Striatal Production



In *Cyathus* spp., biomass production is strongly correlated to striatal production

Fermentations in culture media using soy peptone as nitrogen source gave hitherto unprecedented, rather high yields

Tian Shen, Diploma thesis, JLU Gießen (2009)

C/N ratios and productivity

Medium	SNL	YMG	soy peptone
C-Source	glucose	glucose, malt extract	glucose
N-Source	yeast extract, asparagine	yeast extract	yeast extract, soy peptone
C/N-ratio	10.5:1	10:1	12.8:1

Benefits of soy peptone medium:

- high C/N-ratio → high product yields in mycelia
- soy peptone → essential N-source for biosynthesis
- $CaCl_2$ ⟶ soy peptone, dark cultivation

New results suggest substantially increased productivity as compared to literature data (PhD theses; Univ. Kaiserslautern, in the 1980s and 1990s)

Tian Shen, Diploma thesis, JLU Gießen (2009)

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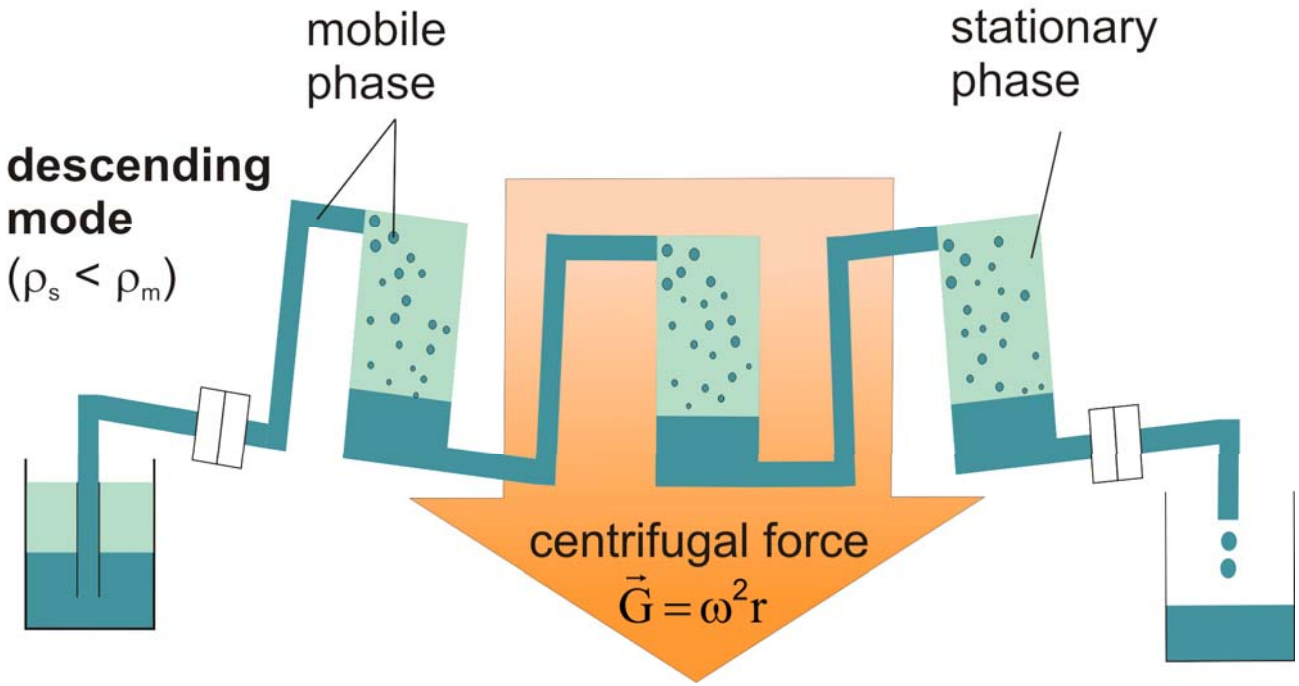
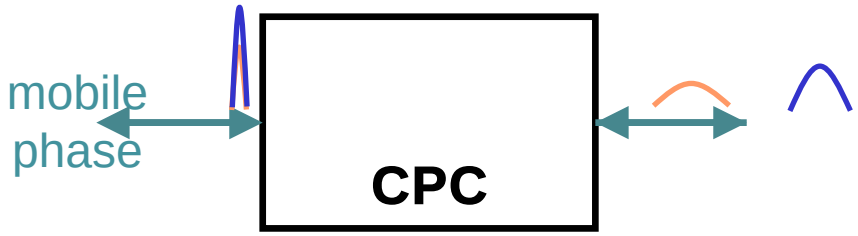
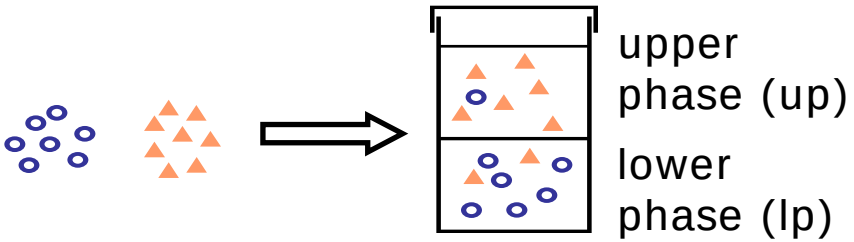
Centrifugal Partition Chromatography (CPC)



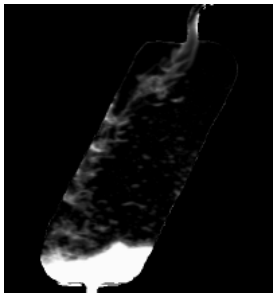


Separation Efficiency in CPC

- **Suitable solvent system**
  - partition coeffcient
  - selectivity factor
  - sample solubility

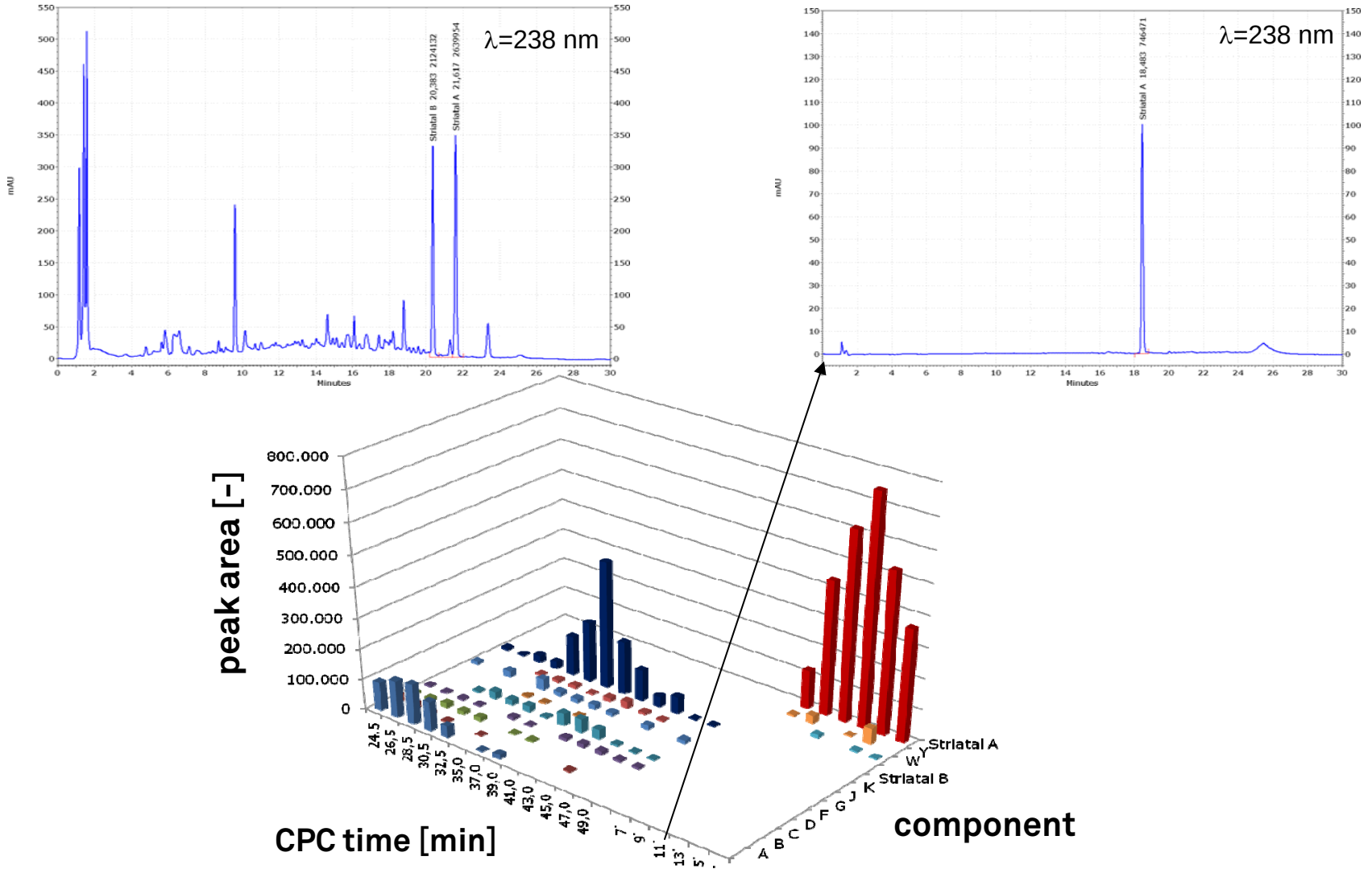


- **Hydrodynamics in chambers**
  - flow pattern
  - degree of dispersion
  - stationary phase hold-up

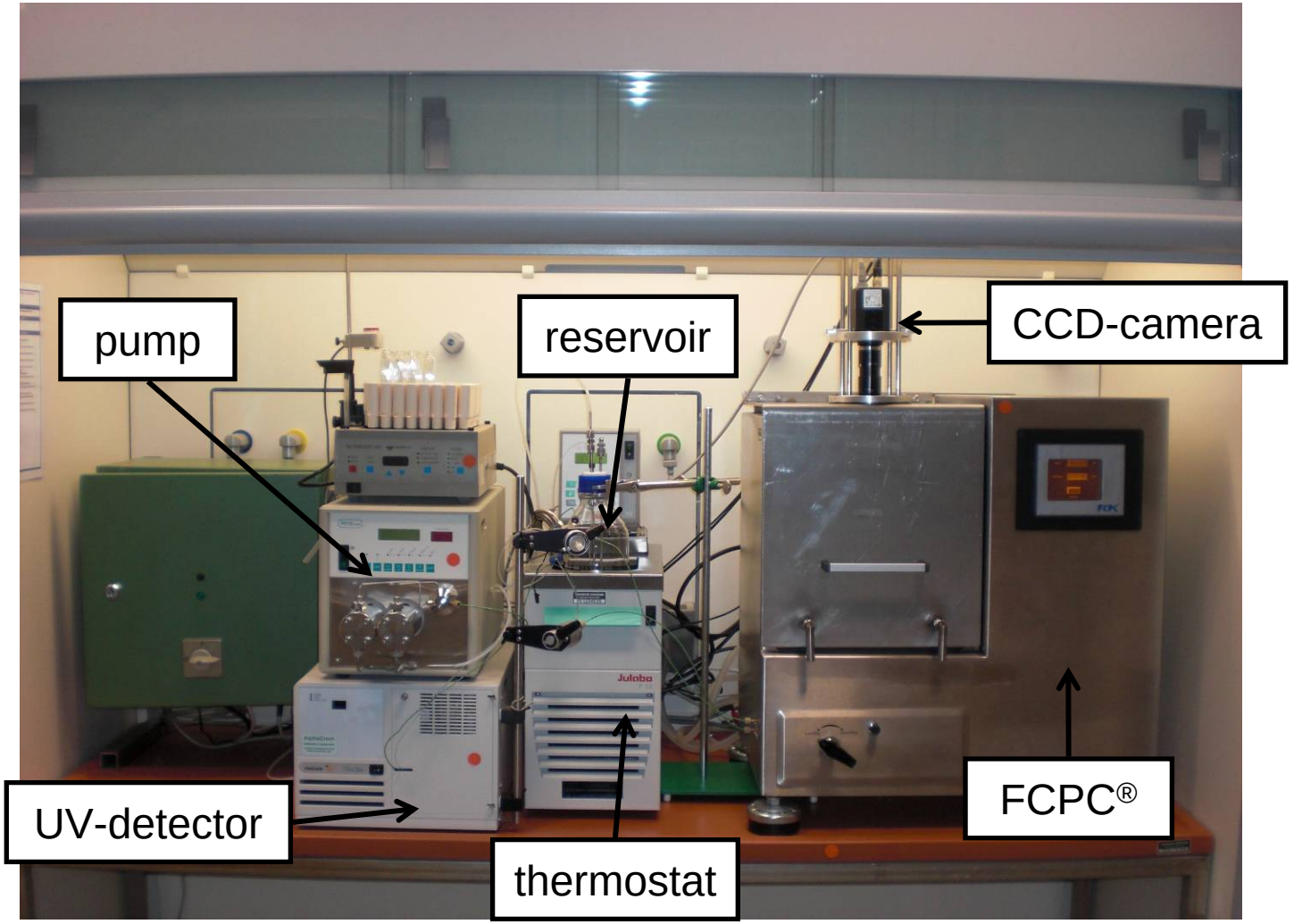


➡ depend on **phase properties** of solvent system and **operating parameters** and

Experiments

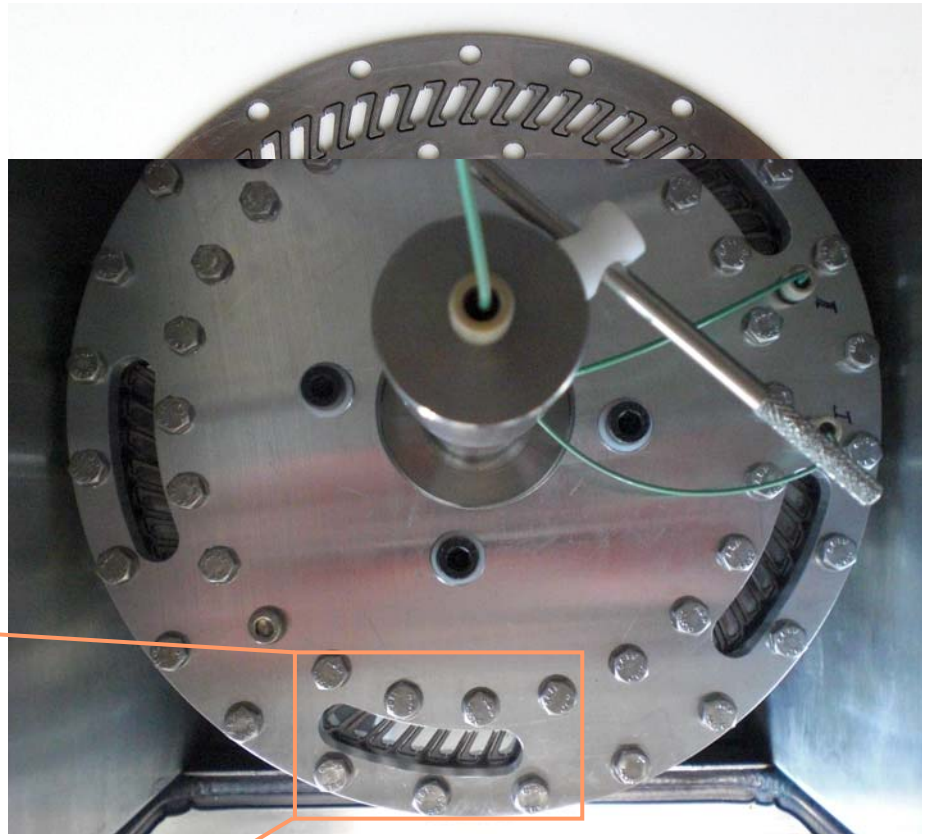


Experimental Setup

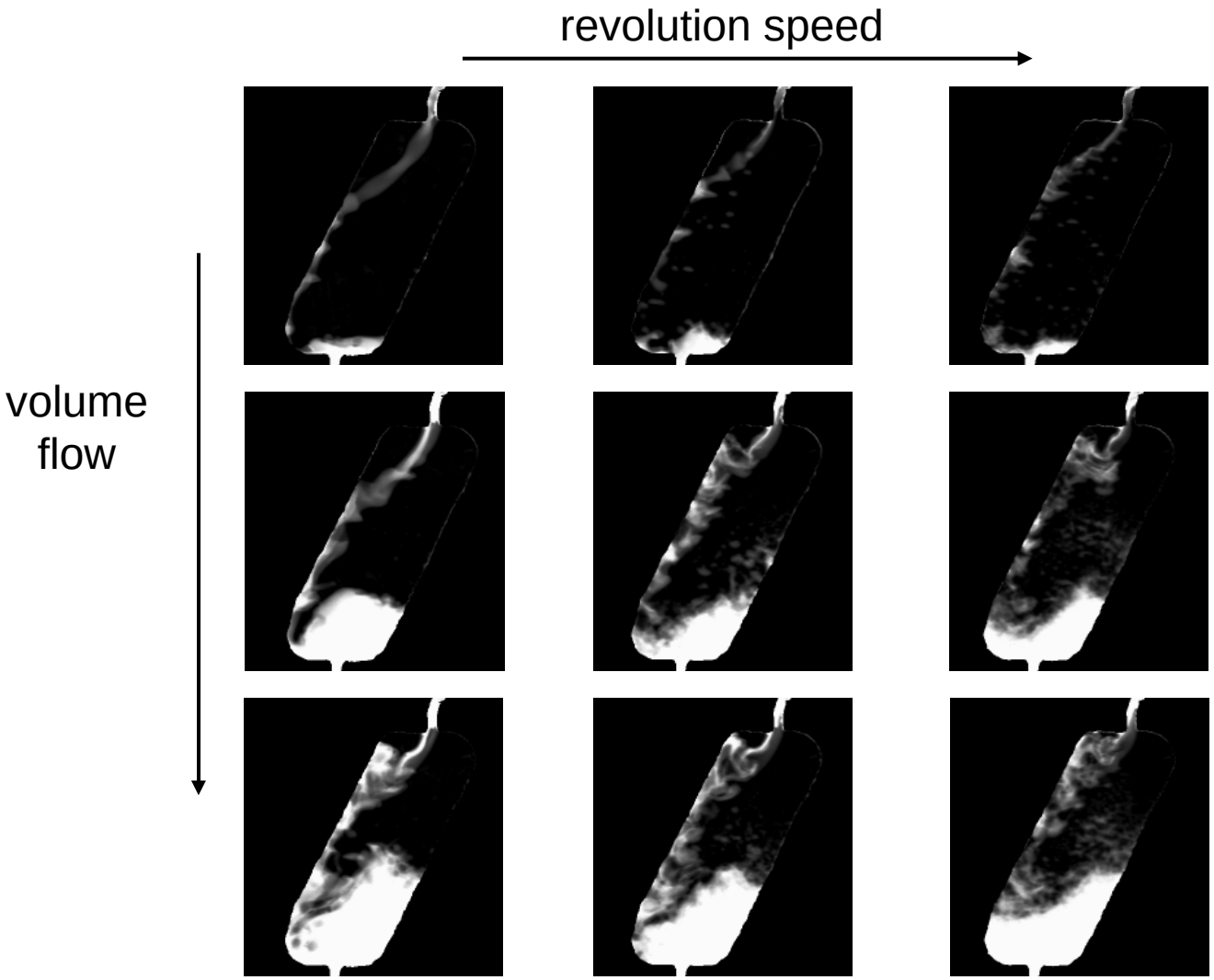


Experimental Setup

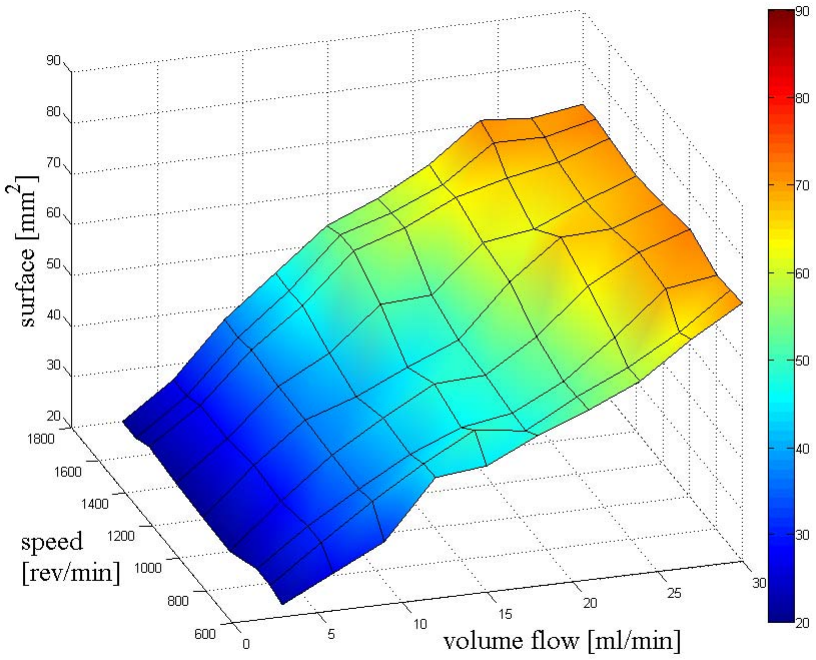
- **Transparent rotor**
  - single FCPC® - plate
  - volume 10 ml
  - sealed with FEP-foil
- **Solvent system**
  - aqueous/organic
  - selective soluble dye



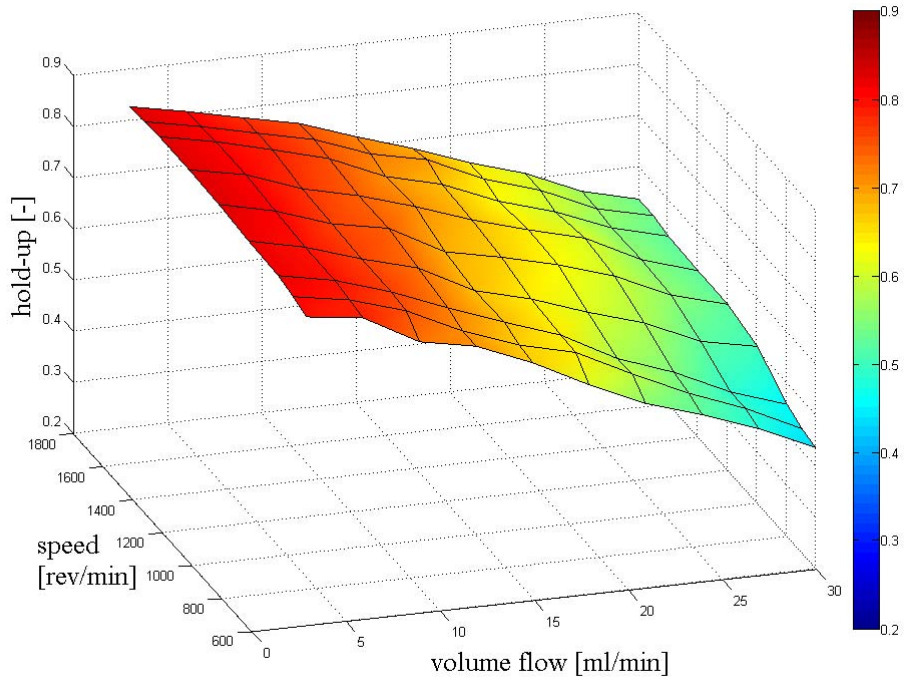
Results



Results interphase area



Results hold-up



Conclusion

- **Hydrodynamics in chambers observable**
- **Strong effect of Coriolis force**
- **Similar flow pattern for both systems**
- **Hold-up and interfacial area**
  - major effect of volume flow
  - minor effect of revolution speed

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Outlook

- **Fermentation technology & microbiological work**
  - *Cyathus*: Further optimisation of fermentation incl. scale-up (30 litre scale)
  - *Hericium* Optimisation of culture media & fermentation processes
  - Biological characterisation of isolated metabolites

- **Analytical & natural product chemistry**
  - Isolation & characterisation of ca. 20 novel compounds detected by HPLC
  - Adapt separation processes to accommodate large amounts of crude material (Goal: availablility of target compounds multi gram scale)

- **CPC technology**
  - Experiments on separation efficiency
  - Optimization of chamber design
  - Validation of multiphase CFD-model

Take Home Message

Interdisciplinary work between

- **academia & industry**
- **engineers, natural product chemists & microbiologists**

➢ **Absolute prerequisite for successful development of innovative methods in biotechnological product development**

Basidiomycetes are still widely unexplored as sustainable sources for useful biologically active compounds, which could change in the near future

Acknowledgments

BMBF & Cluster Industrielle Biotechnologie (CLIB2021) for financial support (Grant No. FKZ 0315404)

CLIB 2021 CLUSTER INDUSTRIELLE BIOTECHNOLOGIE

Projekt 2: Optimierung der Zugänglichkeit mikrobieller Sekundärmetabolite

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