Fermentation-based approaches for the production of biofuels and value-added chemicals

Volha Shapaval (NMBU) and Alexander Wentzel (SINTEF)

12.06.2024

Sub-Project 3.4: Secondary Conversion and Upgrading

WP 2.5 Enzymatic saccharification

KBM

Vincent Eijsink Aniko Varnai Svein Jarle Horn Line Degn Hansen

https://no.pinterest.com/pin/767019380256496400/

Processing,
preteritmet,
hydrolysis

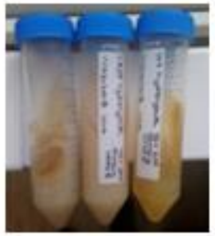
WP 3.4 Fermentation

Realtek SINTEF

Achim Kohler Volha Shapaval Boris Zimmermann Valeria Tafintseva Simona Dzurendova Cristian Bolano Losada Alexander Wentzel Francesca Di Bartolomeo

filamentous fungi

bacteria, yeasts



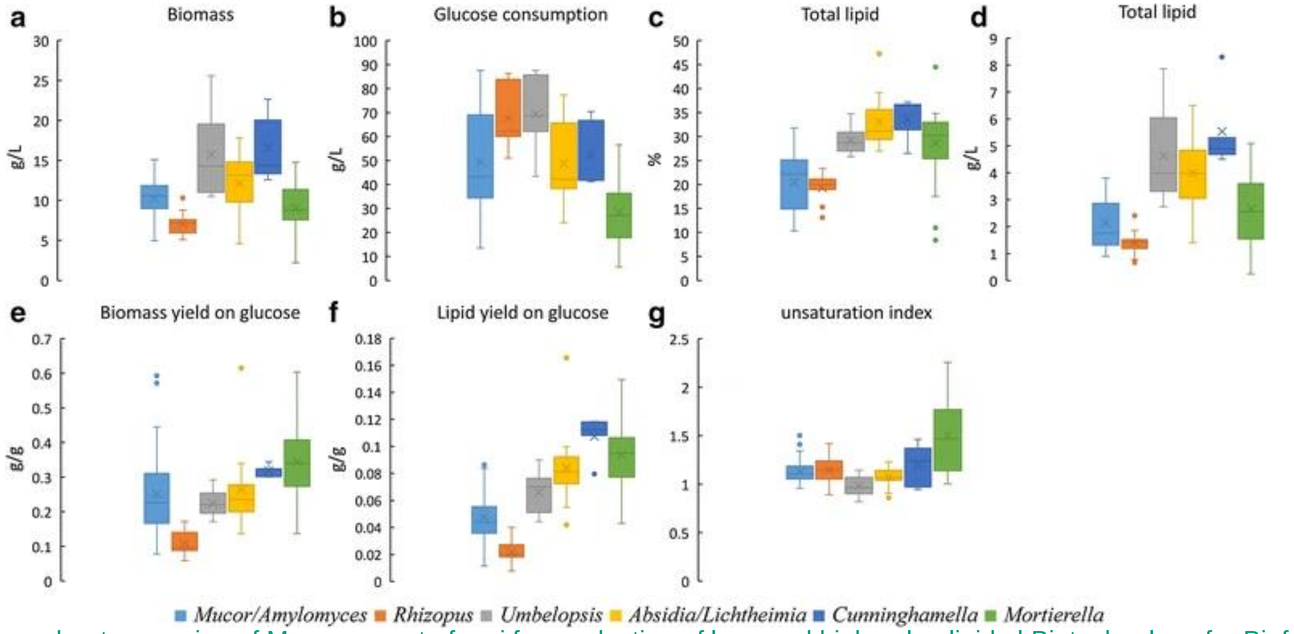
Sugar-rich
hydrolysates

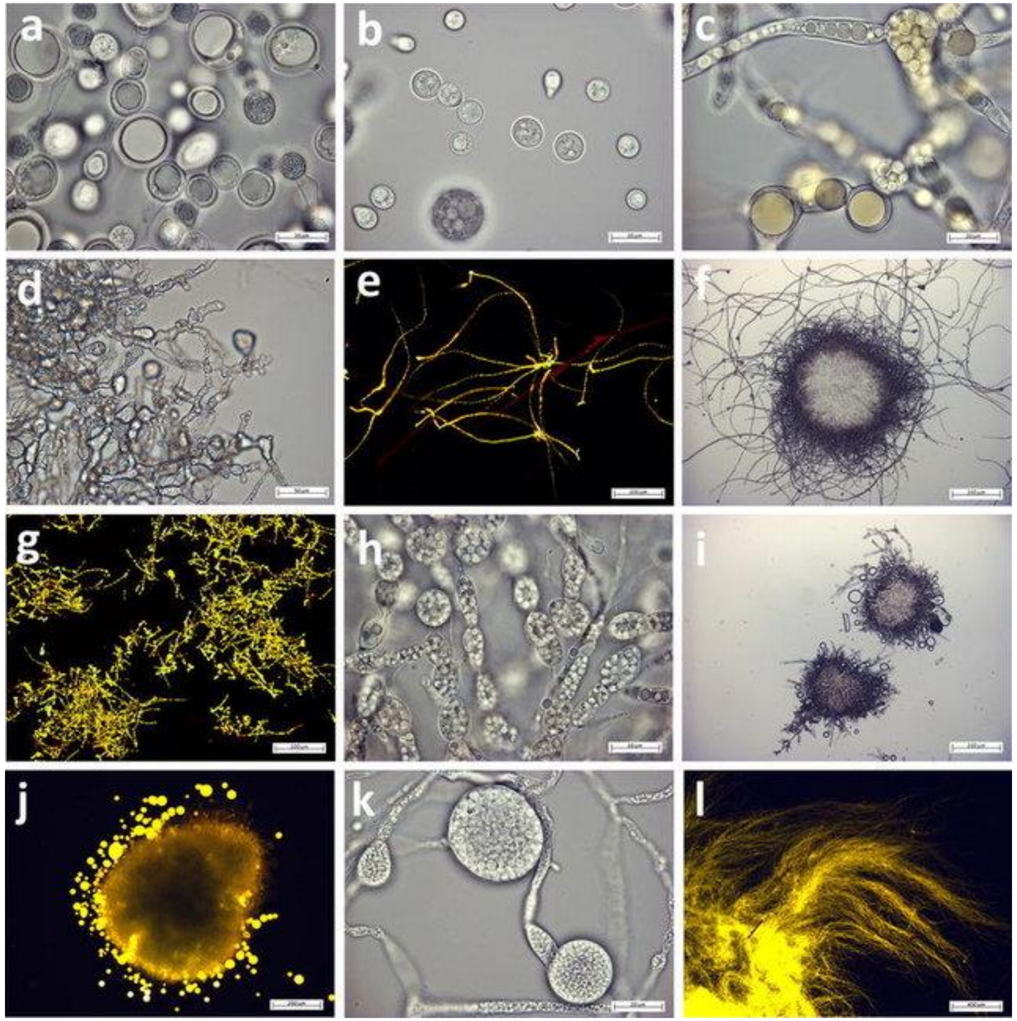
Fermentation

high/low value lipids
biopolymers- chitin/chitosan
polyphosphates
isobutanol
organic acids- butyric acid

Fungal lipids and valuable co-products

Mucoromycota filamentous fungi are able to accumulate large amounts of lipids (up to **70%**). The accumulated lipids are in the form of TAGs and fatty acid profile similar as for plant oils (**omega-6 fatty acids**). Mycoromycota fungi have versatile metabolism and able to convert lignocellulose feedstock. Therefore, these fungi have been positioned as alternative lipid-rich biomass for biofuels.



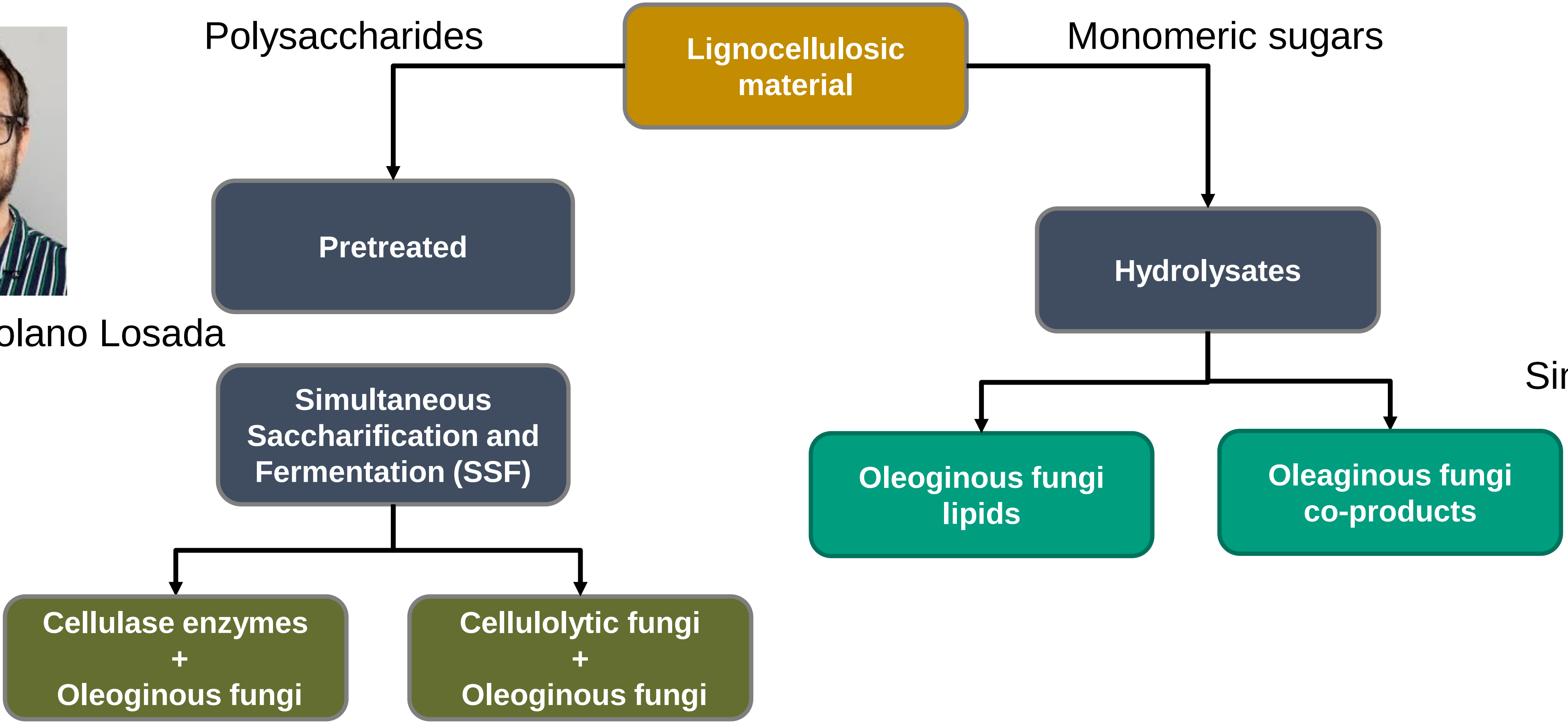


[High-throughput screening of Mucoromycota fungi for production of low- and high-value lipids | Biotechnology for Biofuels and Bioproducts | Full Text (biomedcentral.com)]

[Mucoromycota fungi as powerful cell factories for modern biorefinery | Applied Microbiology and Biotechnology (springer.com)]

Fungal fermentation of lignocellulose material

Cristian Bolano Losada



Simona Dzurendova

Fungal fermentation

Simona Dzurendova

Optimizing production of lipids and valuable co-products in oleaginous Mucoromycota

PhD thesis to be defended in April 2021.

Cristian Bolano Losada

Development of consolidated bioprocess for converting lignocellulose feedstock into fungal lipids.

PhD thesis to be defended in early 2025.

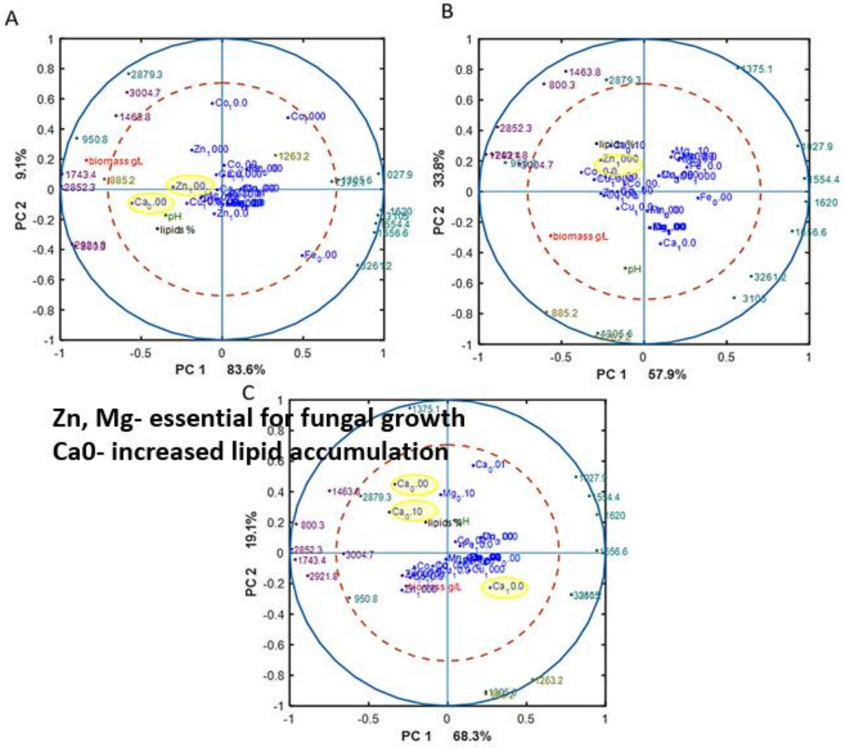
Optimized fermentation substrate to produce lipids and valuable co-products – **Key results**

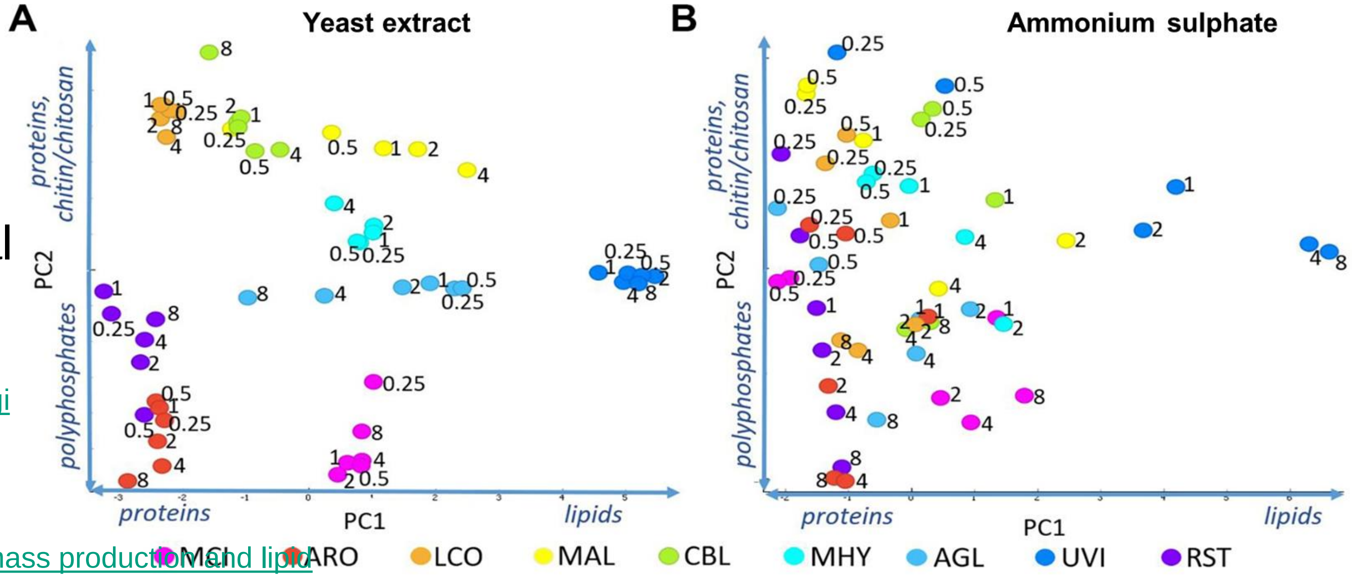
The main valuable co-product produced along with lipids were identified: **chitin/chitosan**, **polyphosphates** and **carotenoids**

Different nitrogen sources have been tested, where **yeast extract** showed to be the most suitable for fungal lipid production

Impact of metal ions was identified to be significant for biomass, lipid and co-products production. **Calcium limitation** showed triggering effect on lipid accumulation for several fungi

Microcultivation in microtiter plates combined with **FTIR spectroscopy** was established allowing high-troughput screening and optimization of fungal lipids and co-products production





[JoF | Calcium Affects Polyphosphate and Lipid Accumulation in Mucoromycota Fungi (mdpi.com)]

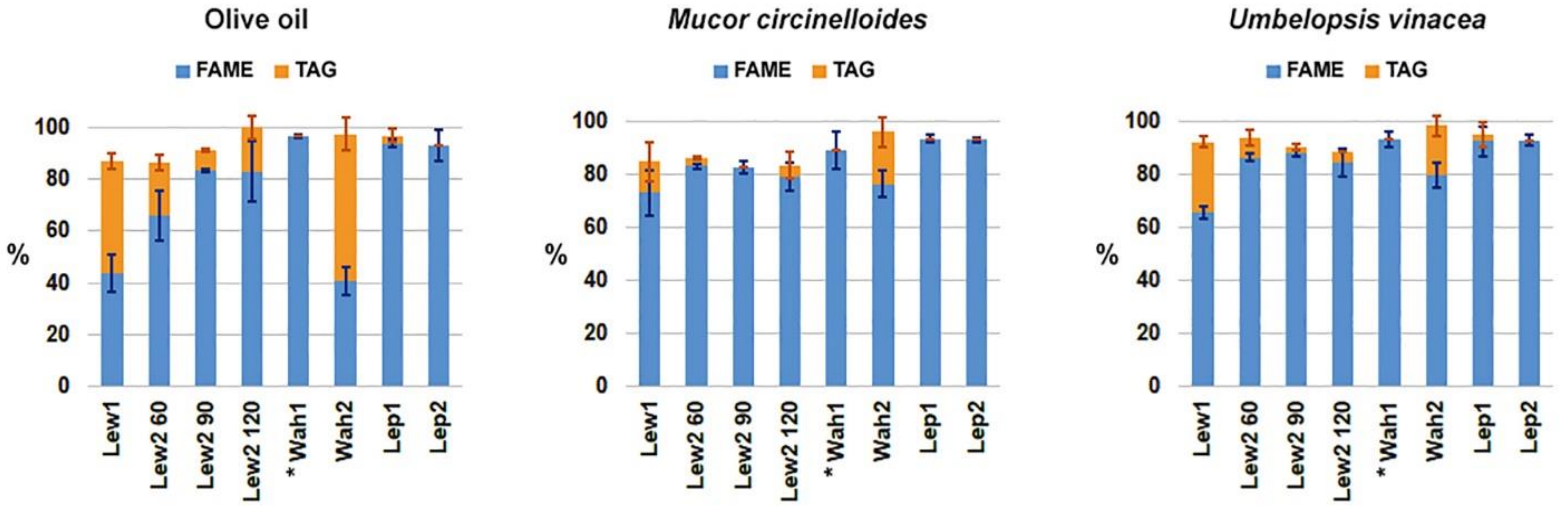
[JoF | Metal and Phosphate Ions Show Remarkable Influence on the Biomass Production and Lipid Accumulation in Oleaginous Mucor circinelloides (mdpi.com)]

[The influence of phosphorus source and the nature of nitrogen substrate on the biomass production and lipid accumulation in oleaginous Mucoromycota fungi | Applied Microbiology and Biotechnology (springer.com)]

[Microcultivation and FTIR spectroscopy-based screening revealed a nutrient-induced co-production of high-value metabolites in oleaginous Mucoromycota fungi | PLOS ONE]

Optimisation of lipid analysis for oleaginous microbial biomass – **Key results**

Transesterification of microbial oils is an essential step in microbial lipid production at both laboratory and industrial scale. Direct transesterification can considerably reduce costs, increase sample throughput and improve lipid yields. The assessment of the direct transesterification methods on a biomass of filamentous fungi was performed.
The evaluation and optimisation of three common direct transesterification methods was performed.



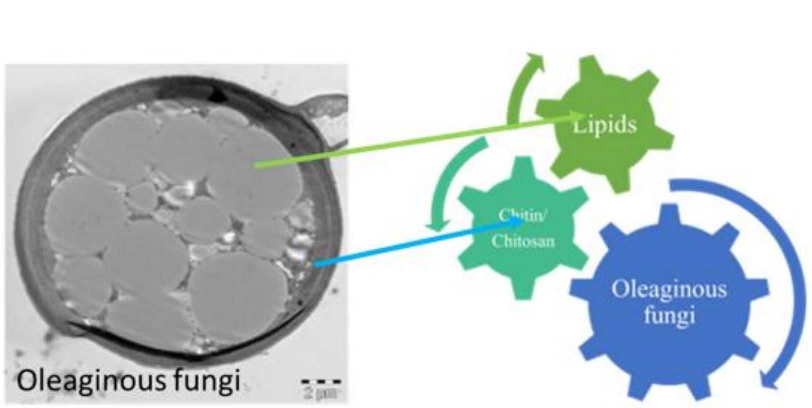
[Evaluation and optimisation of direct transesterification methods for the assessment of lipid accumulation in oleaginous filamentous fungi | Microbial Cell Factories (springer.com)]

Spin-off project on co-production of lipids and chitin/chitosan

NMBU

Main technological objective

To develop **a single fermentation process for co-production of high-value lipids and chitin/chitosan biopolymers** for agricultural applications as animal feed additive and plant biostimulant **from sugar-rich lignocellulose hydrolysates** originating from forestry waste streams.



LIGNOLIPP

Project identity

Funding: Funded within programme Bioeconomy on the North, by the Research Council of Norway (RCN) and the Federal Ministry for Education and Research (BMBF)
Project duration: 2020-2023
Budget: 12 Mio NOK
Partners: 6 partners

Federal Ministry of Education and Research

The Research Council of Norway

BIOECONOMY IN THE NORTH

NMBU

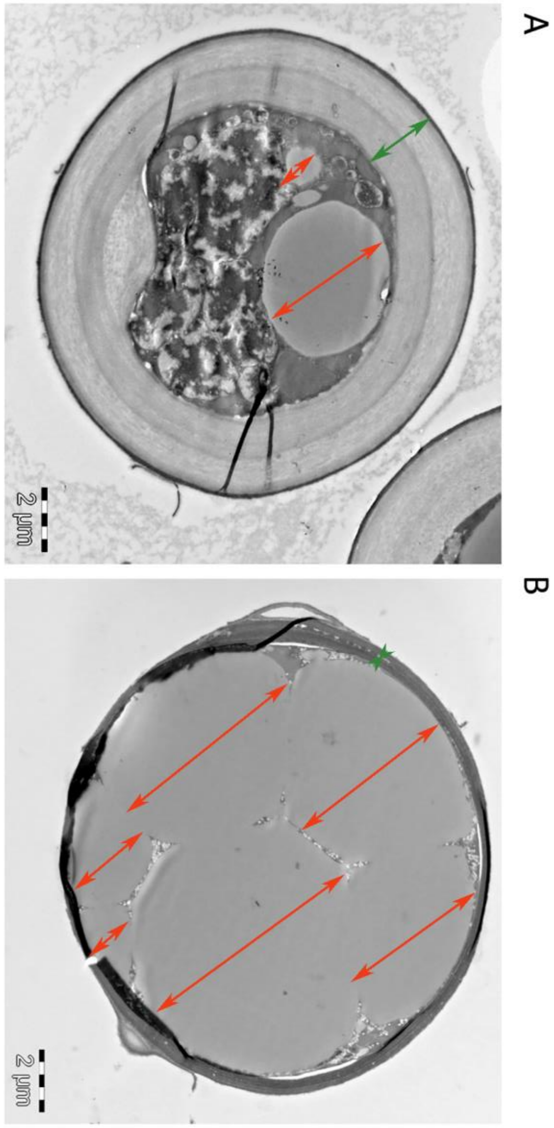
NORCE

Biosentrum AS

Borregaard

Bex-Biotec

Westfälische Wilhelms-Universität Münster



[Microcultivation and FTIR spectroscopy-based screening revealed a nutrient-induced co-production of high-value metabolites in oleaginous Mucoromycota fungi | PLOS ONE]

Strain selection for Simultanuous Saccharification and Fermentation - **Key results**

Relevant characteristics for SSF:
- High lipogenesis activity
- Thermotolerance
- Tolerance to H_2O_2
- Being responsive to cellobiose

Identified the most promising oleaginous strain for SSF

	Lipogenesis (%)	Thermotolerance (°C)	β-glucosidase (U/mL)	Endoglucanase (U/mL)	H_2O_2 tolerance (mM)
C. blakeesleana **CCM F705**	37	35	0	0.85	9.375
M. circinelloides **CCM F220**	23	35	0	0	18.75
M. circinelloides **VI04473**	25	35	0	0	9.375
M. hyalina **VKM F1629**	33	25	0	0	1.172
L. corymbifera **CCM F8077**	**37**	**45**	**0.113**	**1.39**	**4.687**
M. alpina **CBS 4758**	44	30	0	0	n.d.
U. vinacea **CCM F539**	35	30	0	0	1.562
A. glauca **CCM F451**	47	30	0.009	0.59	3.125
R. stolonifer **VKM F400**	21	30	0	0	9.375

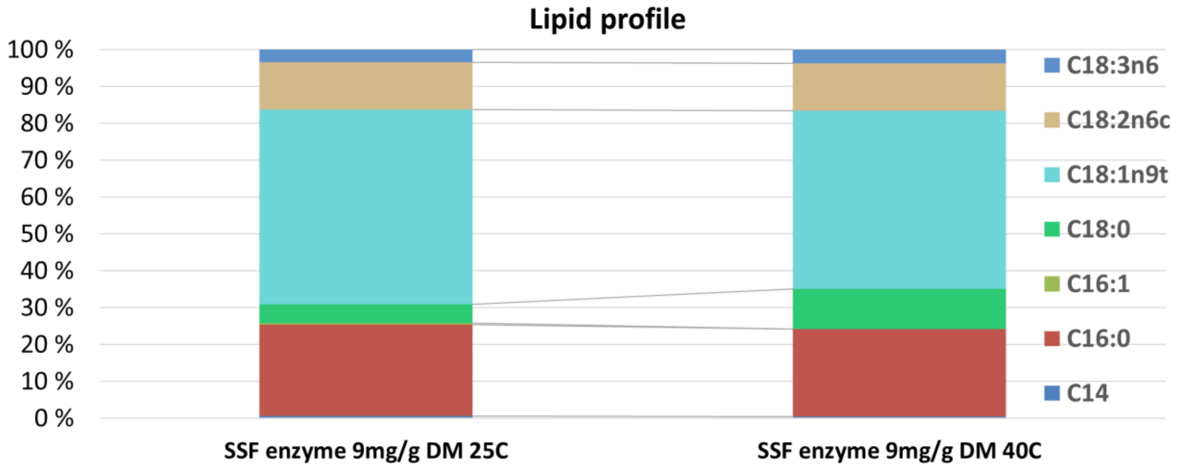


Figure 5. Lipid profile of *L. corymbifera* CCM F8077 grown in SSF process at 25°C and 40°C.

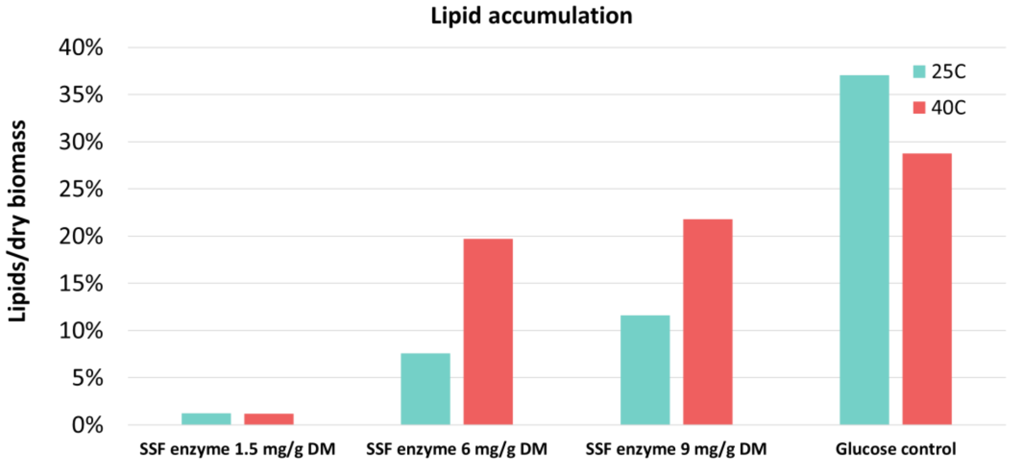
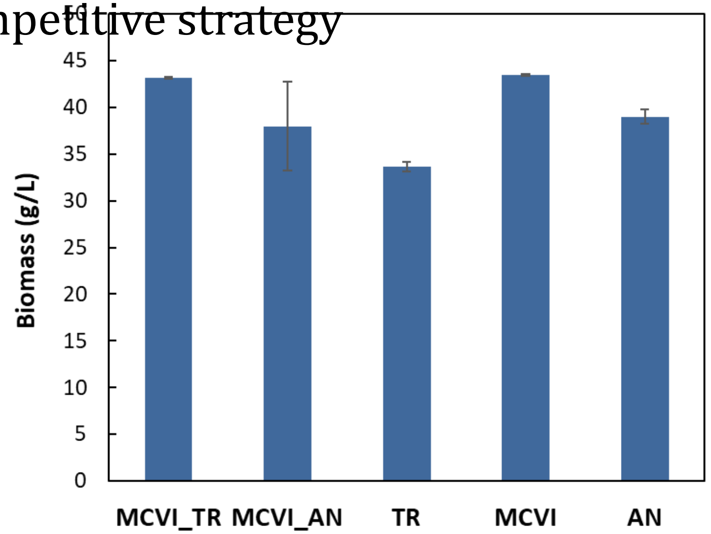


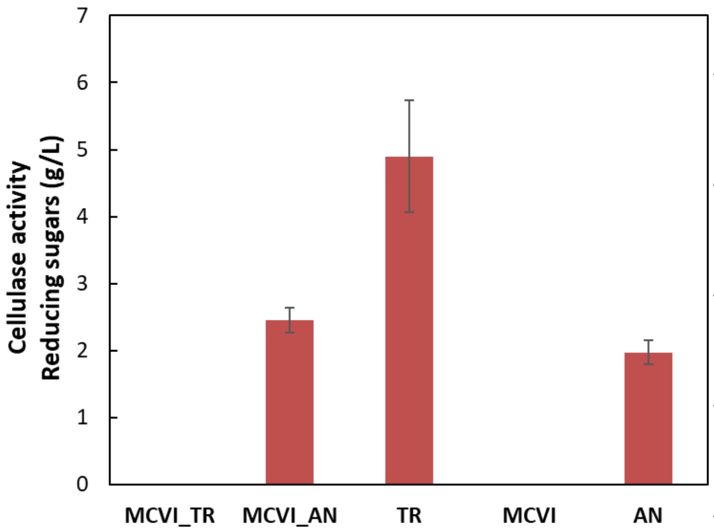
Figure 3. Percentage of lipids in the final biomass (lignocellulose leftovers + lipid-rich fungus biomass) after 8 days of SSF flask scale process and different enzyme dosages.

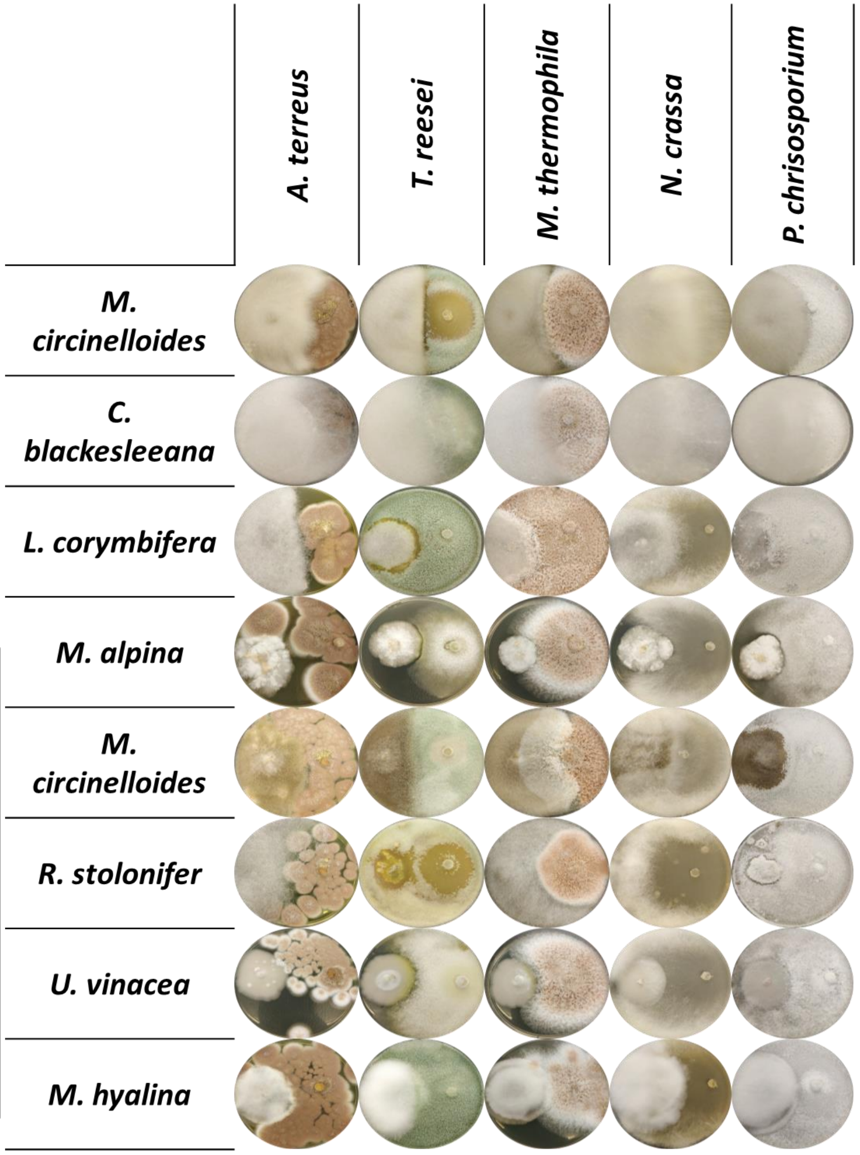
Manuscript 1 and Manuscript 2 in preparation

Confrontation between cellulolytic fungi and oleaginous fungi – **Key results**

Many confronttion experiments have been performed
Two dual species confrantations *Mucor* vs *Aspergillus* and *Mucor* vs *Trichoderma* have been studied in more detials
For *Mucor* vs *Trichoderma* confrontation we found that *Mucor* was unable to produce efficient cellulases while *Trichoderma* expressed cellulase at levels similar to the control, meaning unaffected by the presence of *Mucor*
For Mucor vs *Aspergillus*, ***Aspergillus* overexpressed cellulases** far from the interaction zone with *Mucor*, possibly as a result of a competitive strategy







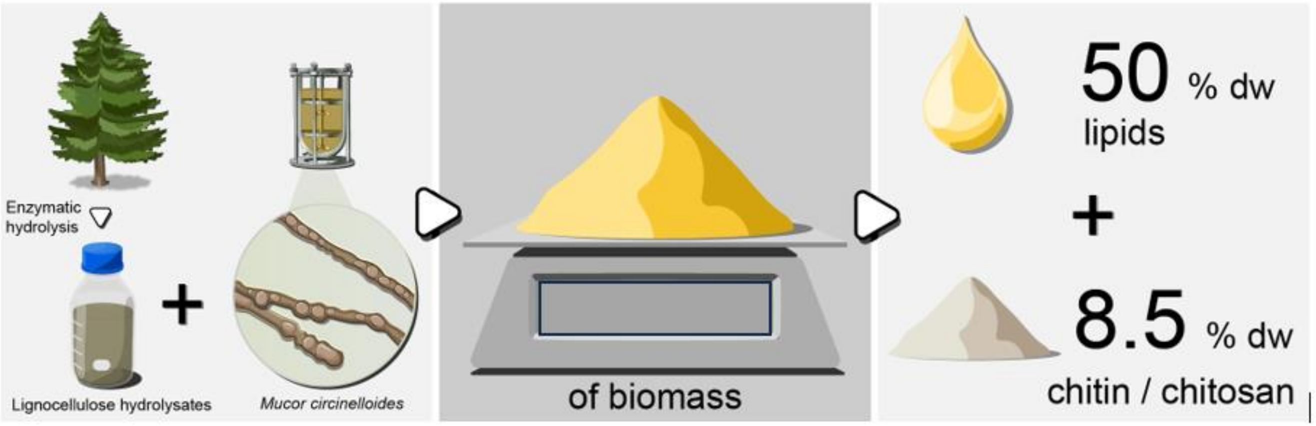
Performance of SSF at 3L bioreactor scale – **Key results**

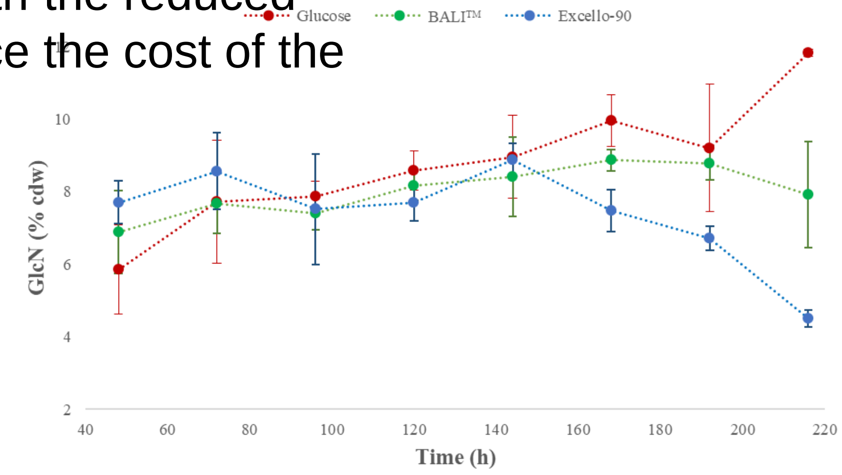
Two fungi, *M.circinelloides* and *L.corymbifera* Involved in the studies.

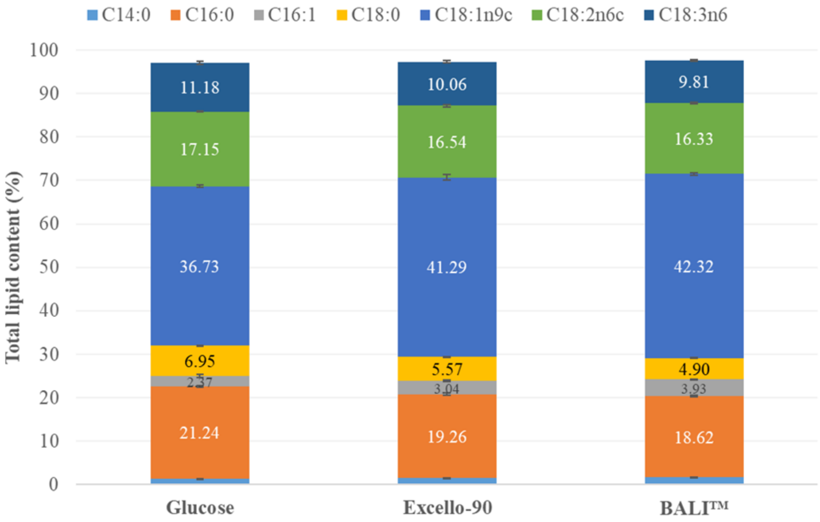
SSF by ***M.circinelloides*** resulted in biomass rich In up to **50% lipids** and upt to **8% of chitin/chitosan**.

The SSF experiments with thermophilic ***L. coymbifera*** were performed with the reduced concentration of enzymes to reduce the cost of the process.

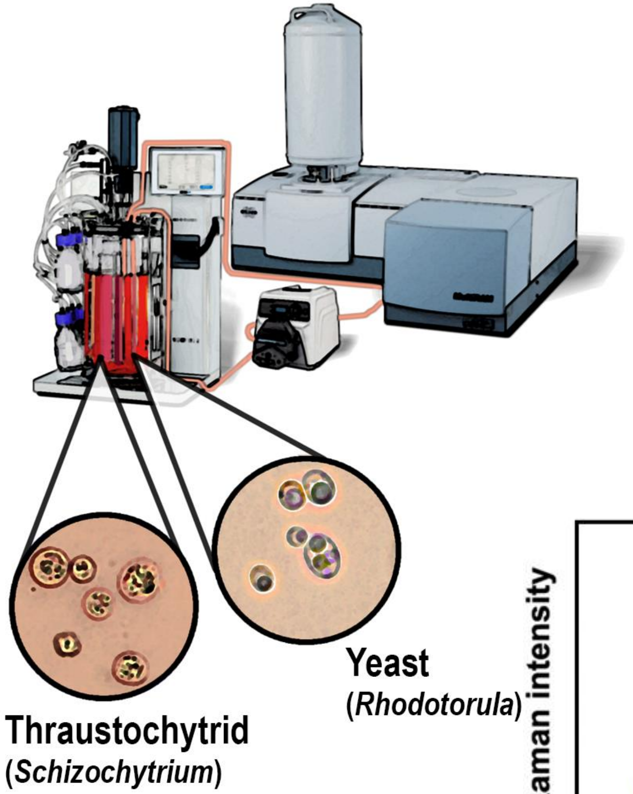
Cultivation of *Mucor circinelloides* on spruce hydrolysates in bioreactor



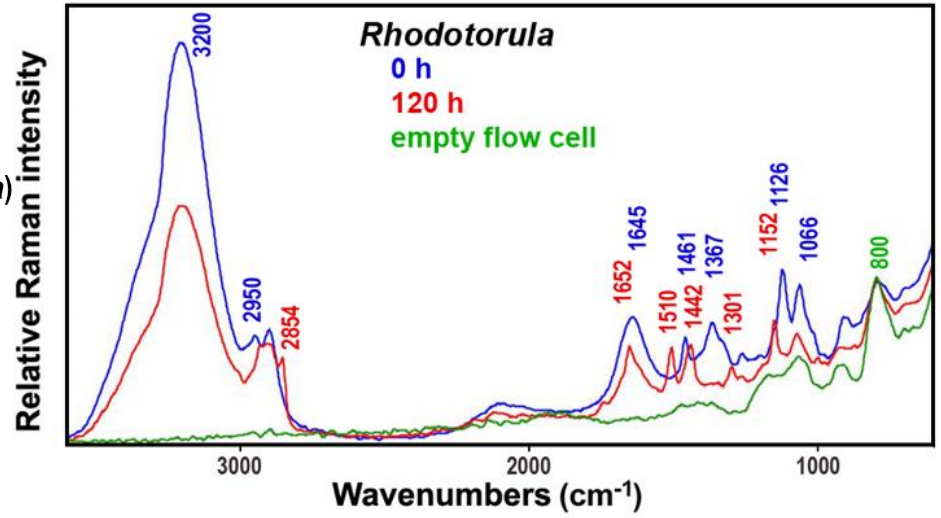




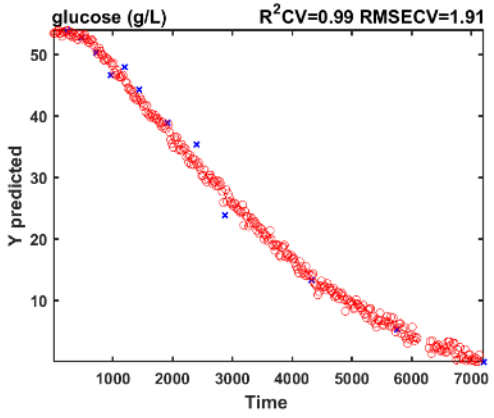
Online fermentation monitoring by vibrational spectroscopy of biomass production, intracellular metabolites, and carbon – **Key results**



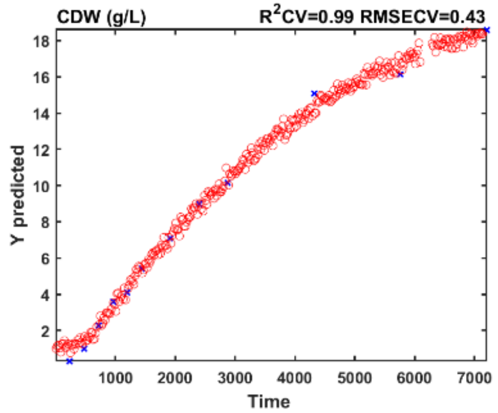
Monitoring of accumulation of lipids and carotenoids in *Rhodotorula toruloides*



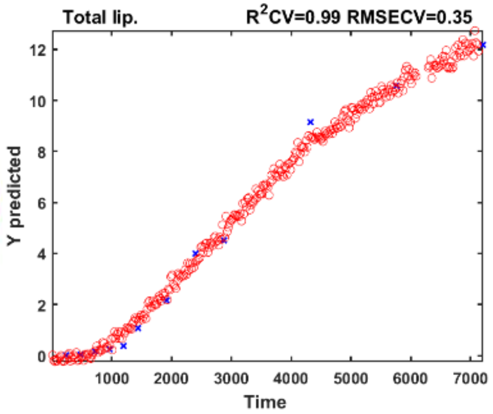
Glucose concentration



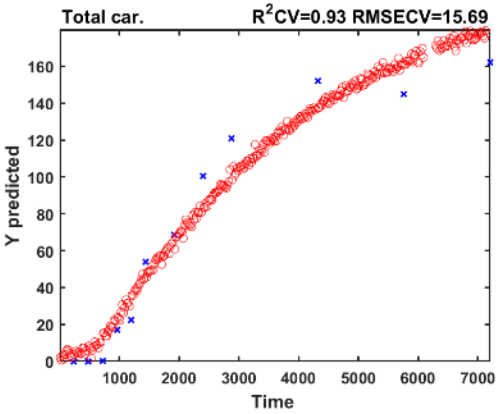
Biomass concentration



Lipids (TAG & FFA) concentration



Carotenoids concentration

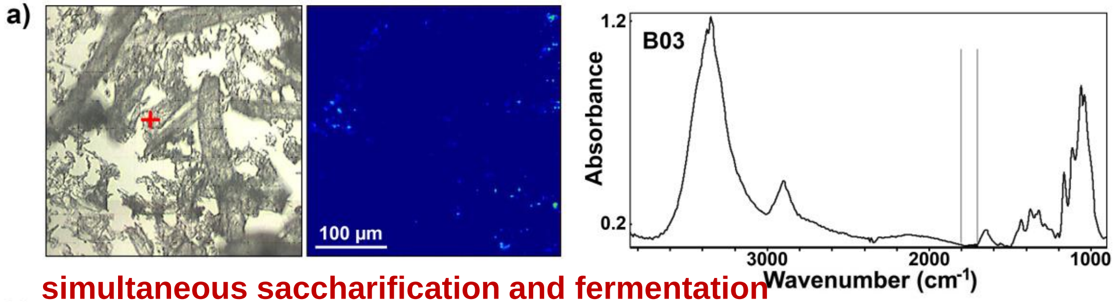


Microb. Cell. Fact. 22 (2023) 261 DOI: 10.1186/s12934-023-02268-y

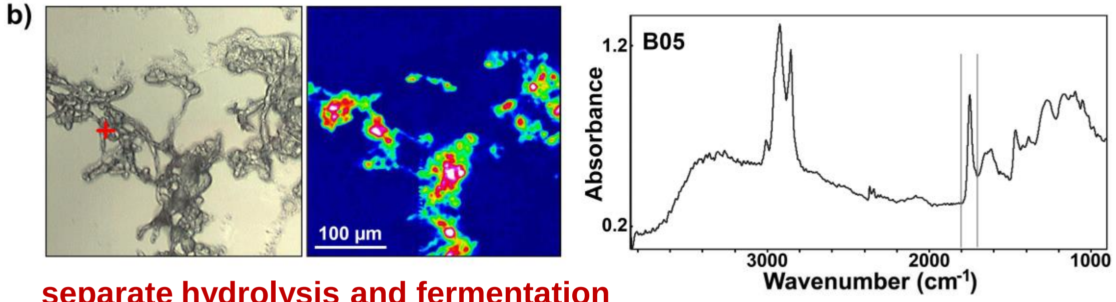
At-line fermentation monitoring by vibrational spectroscopy of biomass production, intracellular metabolites, and carbon – **Key results**

Monitoring of lipid accumulation during simultaneous saccharification and fermentation by oleaginous filamentous fungi

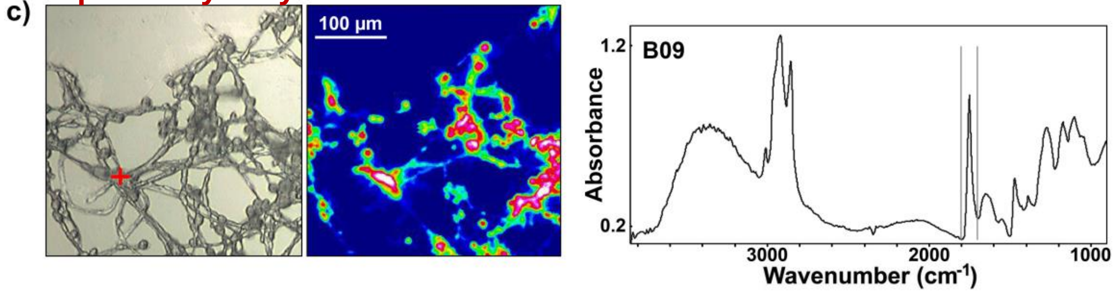
FTIR microspectroscopy of biomass at 216 h from:
lignocellulose control media



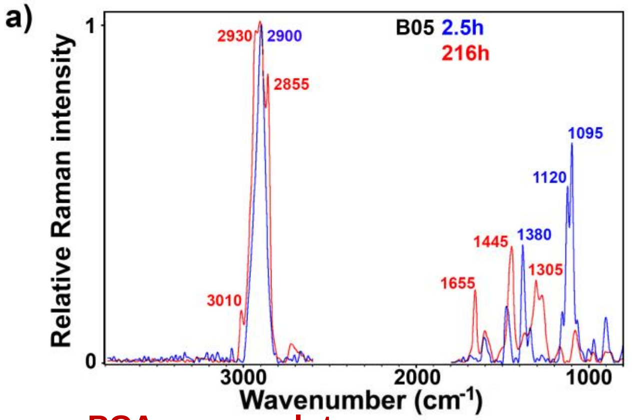
simultaneous saccharification and fermentation



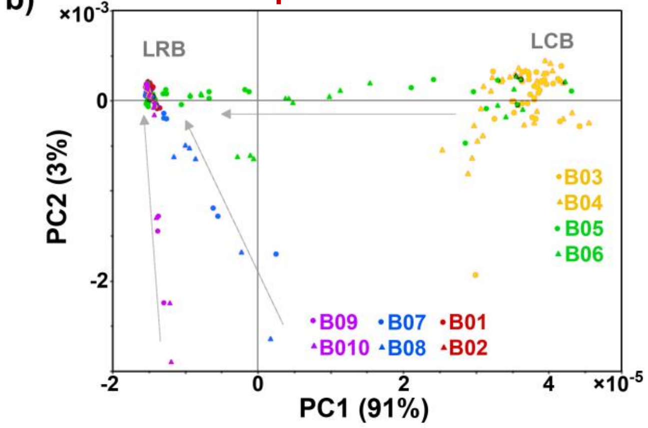
separate hydrolysis and fermentation



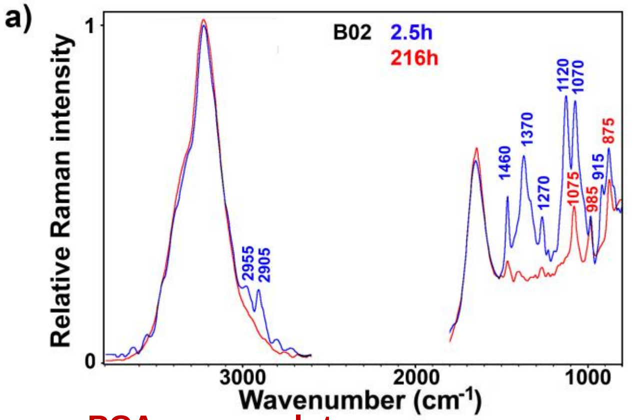
FT-Raman spectroscopy of biomass:



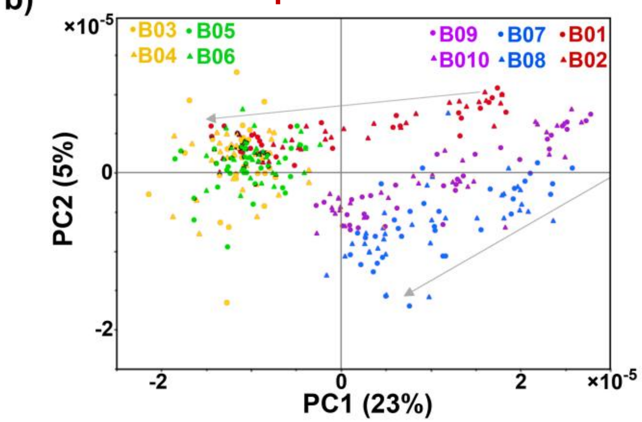
PCA scores plot



FT-Raman spectroscopy of media:



PCA scores plot



Spin-off project on New biobased epoxy compounds for high-performance applications – NordiCoats (2024-2026)

The project's scope encompasses leveraging biotechnological and chemo-synthetic approaches to create safer, potentially recyclable epoxy solutions that significantly reduce reliance on fossil resources. The project's objectives are to innovate in the field of industrial biotechnology, creating new knowledge, methodologies, and competences that contribute to the green transition in the chemical and materials sector, while promoting the development of more sustainable industrial products with a lower environmental footprint.

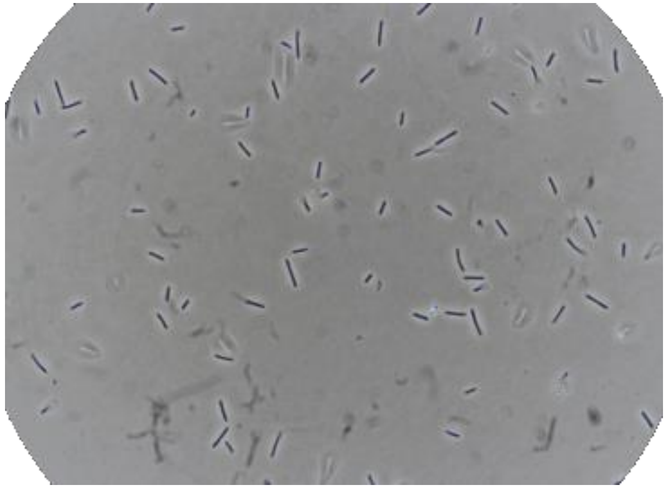
Project partners

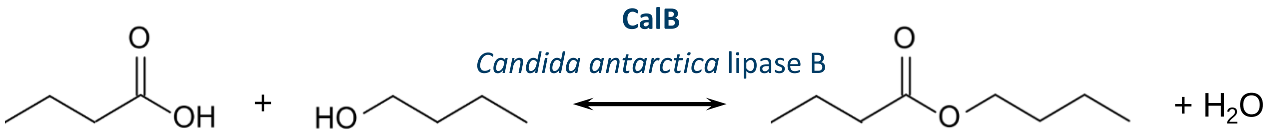
- SINTEF Industry (project owner)
- SINTEF Community
- SINTEF Ocean
- NMBU
- RISE PFI
- Arbaflame AS
- AlgiPharma AS
- BIoenvision AS
- Jotun AS

NordiCoats

Clostridial production of alcohols, acids, and esters

- **Clostridia acetogens** convert biomass-derived sugars into organic acids, alcohols, and solvents (e.g. acetic/butyric acid, acetone-butanol-ethanol (ABE)) under anaerobic (oxygen-free) conditions.
- By applying lipase enzymes as **biocatalysts** and **process integration** (fermentation-separation, e.g. co-extraction into non-polar solvent), the acids and alcohols can be condensed into **carboxyl esters** like **butyl butyrate for use in diesel (and potentially jet)** engines or in flavours & fragrances.



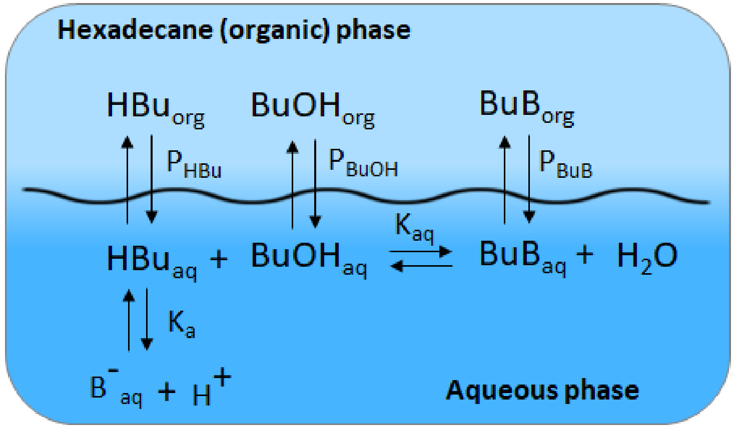


HBu (butyric acid)
Organic acid

BuOH (1-buanol)
Alcohol

BuB (butyl butyrate)
Ester

- The use of **thermophilic Clostridia**, growing at temperatures between 55 and 65°C, increases the overall productivity and reduces the risk of contamination (e.g. from unsterile feedstock).

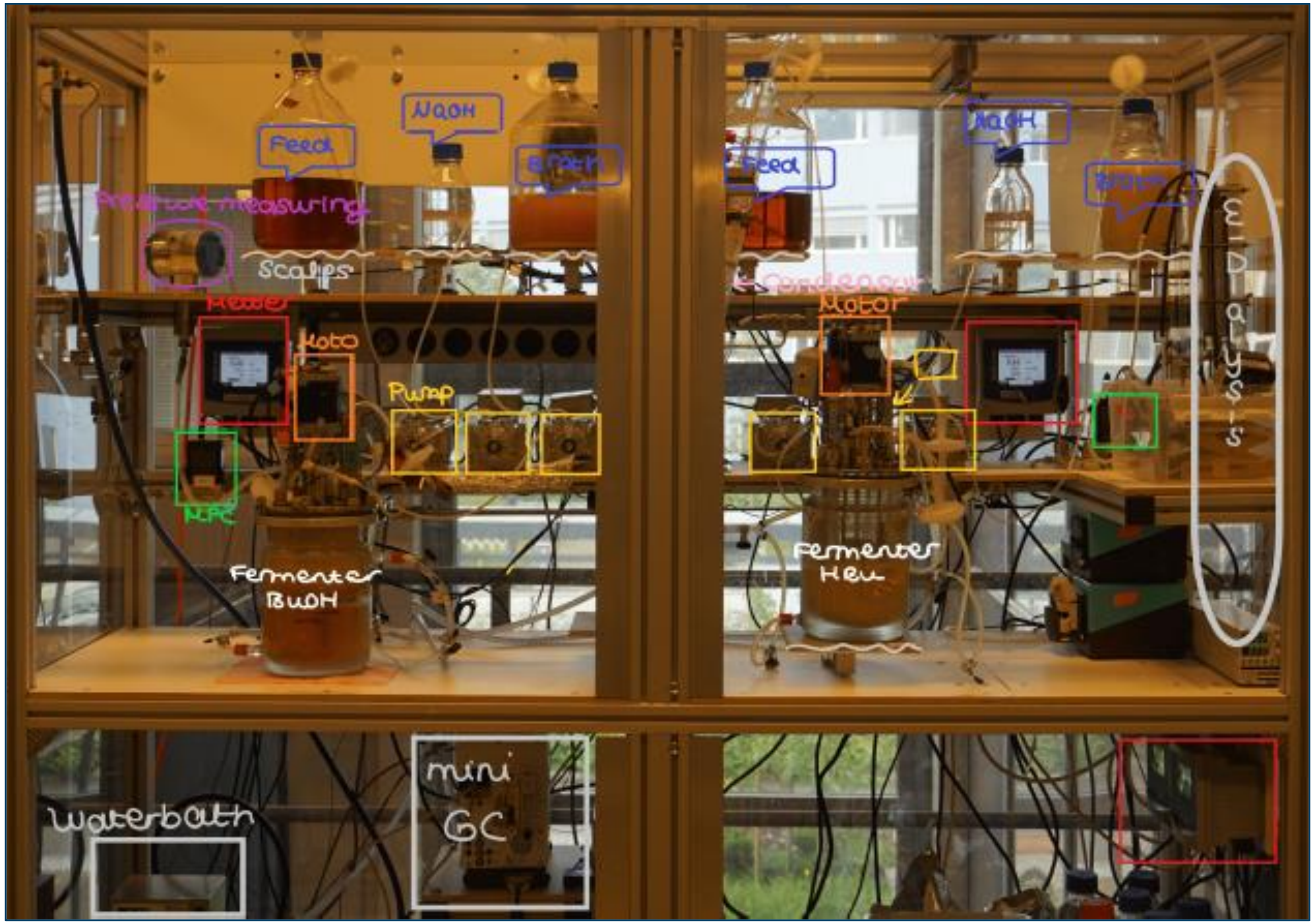


(Modified from: Van den Berg, *et al.*,2013, DOI: 10.1002/bit.24618)

Clostridial production of alcohols, acids, and esters

Key Bio4Fuels results:

- A **new strain collection** of 10+ different thermophilic Clostridia strains has been generated and respective fermentation conditions established.
- A **NTNU/SINTEF bioprocess rig** (TRL5) for the parallel clostridial production of butyric acid and butanol and their subsequent esterification has been established.



Bio4Fuels/EcoLodge process rig at SINTEF/NTNU. Picture taken from Student Internship Report 2022. Interns: Marla Braun, Simon Kunz, Anna Rieck. Supervision: Henri Steinweg (IKFT, KIT), Bernd Wittgens and Theresa Rücker (SINTEF).

Clostridial production of alcohols, acids, and esters

Key Bio4Fuels results:

- **Extended continuous cultivations** of (A) *Clostridium beijerinckii* for butanol production and (B) *Clostridium tyrobutyricum* for butyric acid production using the NTNU/SINTEF Bio4Fuels/EcoLodge process rig.

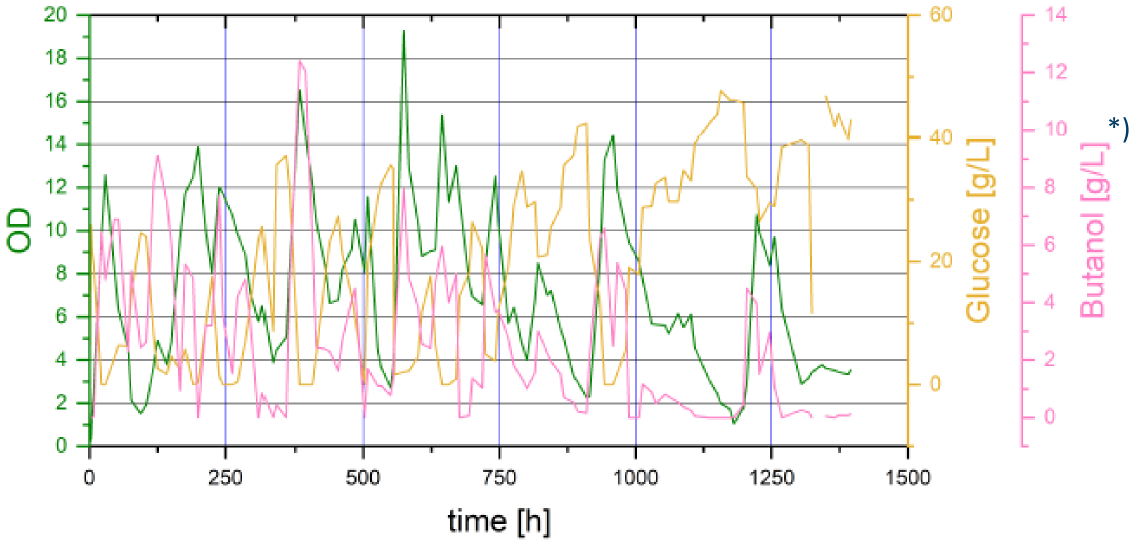
A

Parameter	Unit	Glucose	Acetone	Butanol	Ethanol
Turnover	[%]	76.6	-	-	
Selectivity	[mol/mol]	-	0.090	0.237	0.034
Yield (Mol)	[mol/mol]	-	0.069	0.182	0.026
Yield (Carbon)	[C/C]	-	0.045	0.158	0.023
Space Time Yield	[g/L*h]	-	0.025	0.083	0.015
Glucose uptake rate	[g/h]	2.33	-	-	-

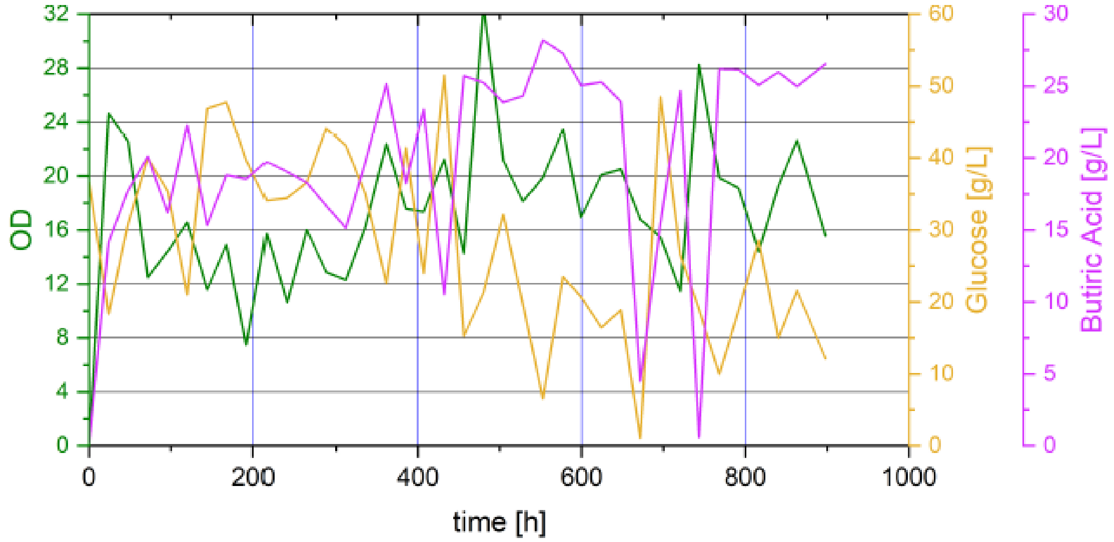
B

Parameter	Unit	Glucose	Butyric Acid	Acetic Acid
Turnover	[%]	72.2	-	-
Selectivity	[mol/mol]	-	0.587	0.078
Yield (Mol)	[mol/mol]	-	0.424	0.057
Yield (Carbon)	[C/C]	-	0.284	0.038
Space Time Yield	[g/L*h]	-	0.33	0.12
Substrate uptake rate	[g/h]	2.33	-	-

A



B



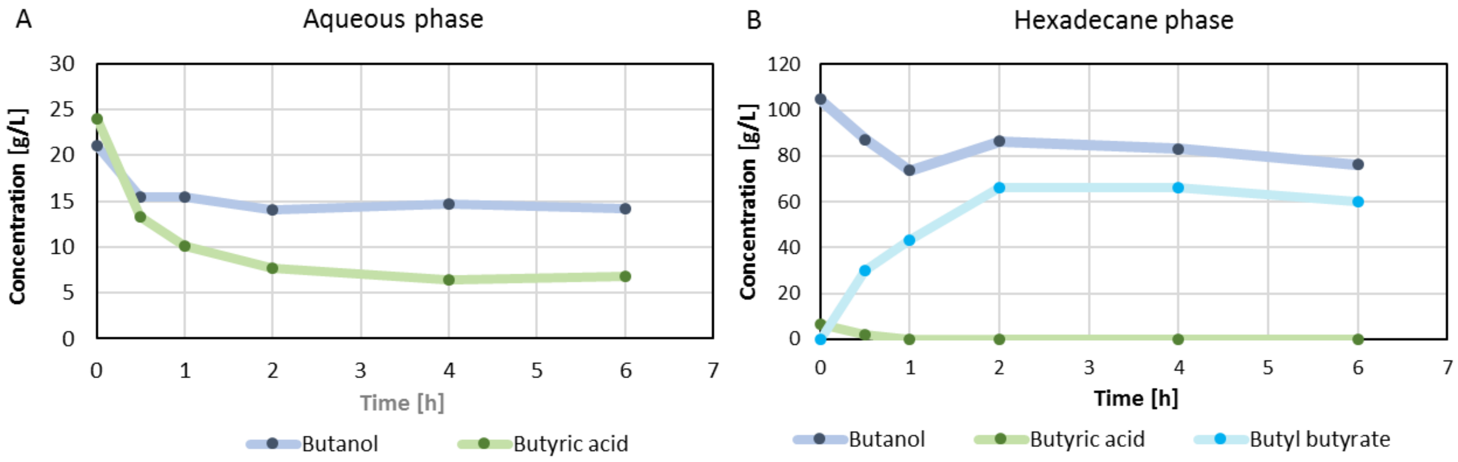
*) Most of the butanol (ABE) is recovered from the off-gas condensate by gas stripping.

Pictures and data taken from Student Internship Report 2022. Interns: Marla Braun, Simon Kunz, Anna Rieck. Supervision: Henri Steinweg (IKFT, KIT), Bernd Wittgens and Theresa Rücker (SINTEF).

Clostridial production of alcohols, acids, and esters

Key Bio4Fuels results:

- **Enzymatic esterification** using butanol (BuOH) and butyric acid (HBu) and simultaneous extraction of the ester into **butyl butyrate (BuB)** has been extensively studied in detail.
- **A number of spin-off/parallel projects have been established**, i.e., RCN IndNor EcoLodge and NanoLodge (NTNU (COO)/SINTEF); ERA-CoBioTech BESTER and EU Green Deal project PYROCO$_2$ (SINTEF COO).

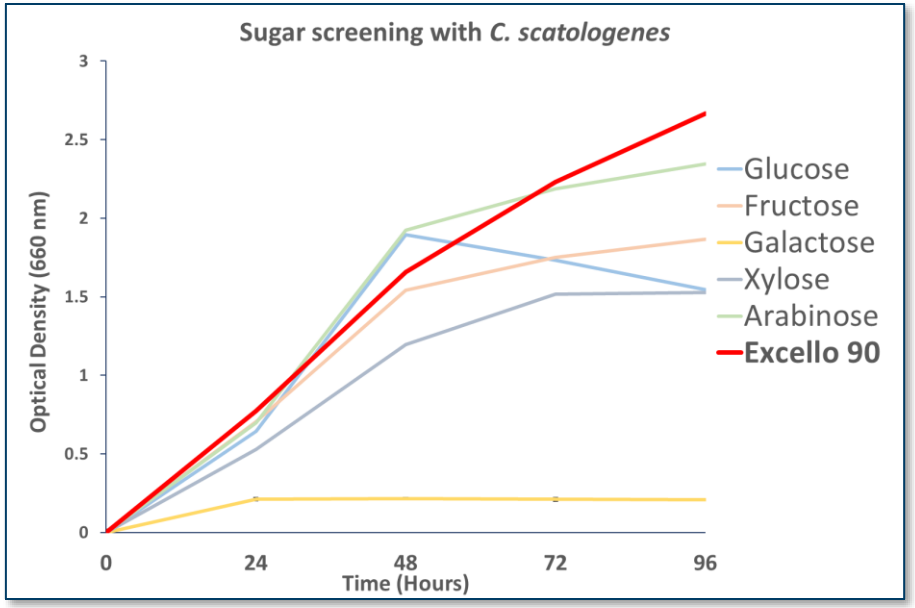


Conversion of BuOH and HBu to BuB in a water-hexadecane 2-phase system. Concentrations of HBu, BuOH and BuB in the aqueous (A) and hexadecane (B) phase measured by Gas Chromatography Mass Spectroscopy (GC-MS). Process conditions: T=35°C; HBu concentration: 20 g/L; BuOH : HBu ratio: 3 : 1 (3-fold excess of BuOH corresponding to 60 g/L); water : hexadecane ratio: 2:1; Enzyme: Novozym 435 at 20 g/L (immobilized on acrylic resin).

- ➢ **Activities currently being continued in the RCN IndNor project NanoLodge**
- ➢ **Scientific manuscript in preparation**

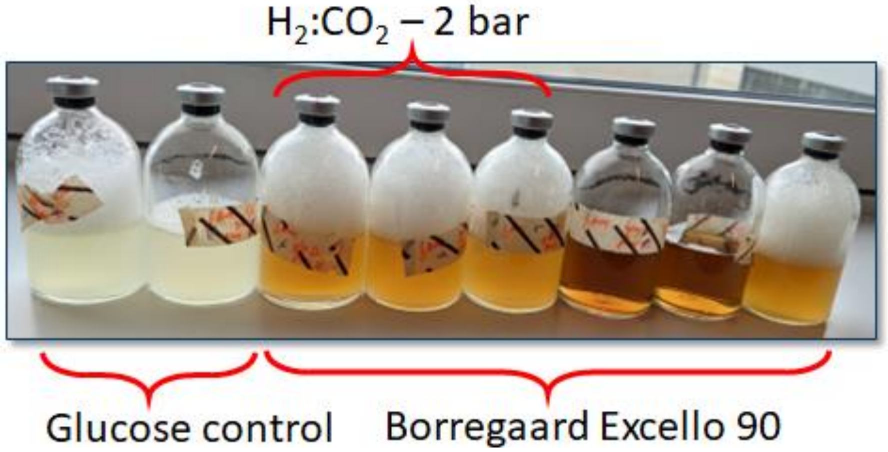
Clostridial mixotrophy: Co-utilization of sugars and CO_2

- <u>Latest focus:</u> Some acetogenic Clostridia are capable of co-utilizing sugars and CO_2 by means of **mixotrophy.** Co-feeding CO_2 (e.g. from industrial processes) to cultures of such strains can lead to better carbon utilization in the synthesis of the bio-based fuels and chemicals.



Sugar utilization by *Clostridium scatologenes*.The strictly-anaerobic bacterium *C. scatologenes* can utilize different sugars as carbon sources. To assess the sugar substrate spectrum, *C. scatologenes* was grown on several different sugars. The crude Excello 90 from Borregaard's BALI process was wound to be a suitable feedstock for *C. scatologenes*, in terms of growth and fermentative performance.

Mixotrophy of *Clostridium scatologenes* in serum flasks. The strictly-anaerobic bacterium *C. scatologenes* can co-utilize sugars and CO_2 as carbon sources. A gas-exchanger (left) is used to precisely apply defined mixtures of H_2:CO_2 (e.g. 80:20) into serum flasks (below) with *C. scatologenes* to assess carbon utilisation via product formation/gas consumption. Favourable gas consumption was ontained with Excello 90 as the sugar substrate supplement. Particularly good growth was observed in the presence of Excello 90 and H_2:CO_2 gas. Transfer to gas fermenters is currently ongoing.

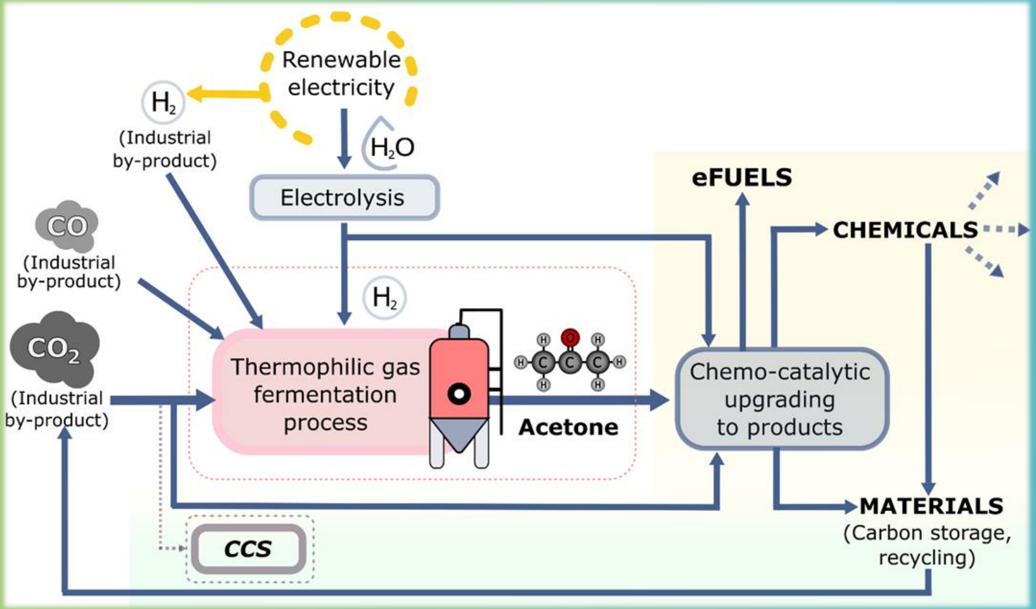


PYROCO$_2$

<u>Spin-off project</u>: PYROCO$_2$ - Demonstrating sustainable value creation from industrial CO_2 by its thermophilic microbial conversion into acetone

PYROCO$_2$ will demonstrate the scalability and economic via-bility of carbon capture and utilization (CCU) using the inno-vative PYROCO$_2$ bioprocess.

The **PYROCO$_2$** demonstrator plant will have a capacity to produce up to 4,000 tonnes acetone annually from 9,100 tonnes of industrial CO_2 and green hydrogen, as a platform for manufacturing eFuels, chemicals, and materials for a wide range of markets.



The demonstrator will be located at the industrial cluster of Herøya Industrial Park in southern Norway. From here, the **PYROCO$_2$** project will represent a key driver for the emergence of CCU Hubs across Europe.

Horizon 2020 call: LC-GD-3-1-2020
Project type: Innovation Action (IA) – TRL 4/5 to 7
Project period: 60 months, 10/2021 to 03/2027
Total project budget (EC contribution): 43 (40) million €

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101037009.

More info: www.pyroco2.eu

NMBU