BIOCON-CO_2

Microbial cell factories and progress towards producing target products

Final symposium 14th of June

Dr. Gabriele Philipps

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This project has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement no. 761042 (BIOCON-CO_2). This output reflects the views only of the author(s), and the European Commission cannot be held responsible for any use which may be made of the information contained therein.

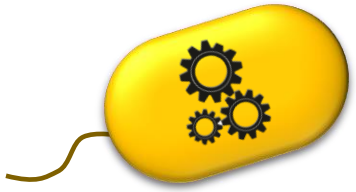
Recycling of Industrial Process Gas for Synthesis of Bulk Chemicals

BIOCON-CO_2

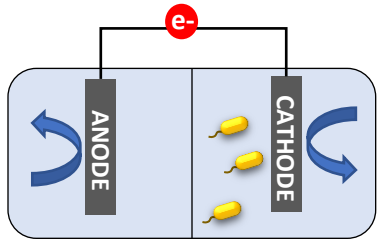
e.g. steel industry

Syngas

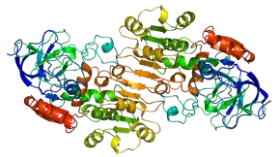
(CO, **CO_2** & H_2)



Engineered microbes



Electrofermentation



Isolated enzymes

Biochemicals

- C3-C6 alcohols

(isopropanol, butanol, hexanol)

- 3-HP
- Formic acid
- Lactic acid

https://pngimage.net/chemie-png-7/

Metabolic engineering of *C. ljungdahlii* for production of Butanol and Hexanol on CO_2 and H_2

Dr. Gabriele Philipps

M.Sc. Ira Lauer

Dr. Stefan Jennewein

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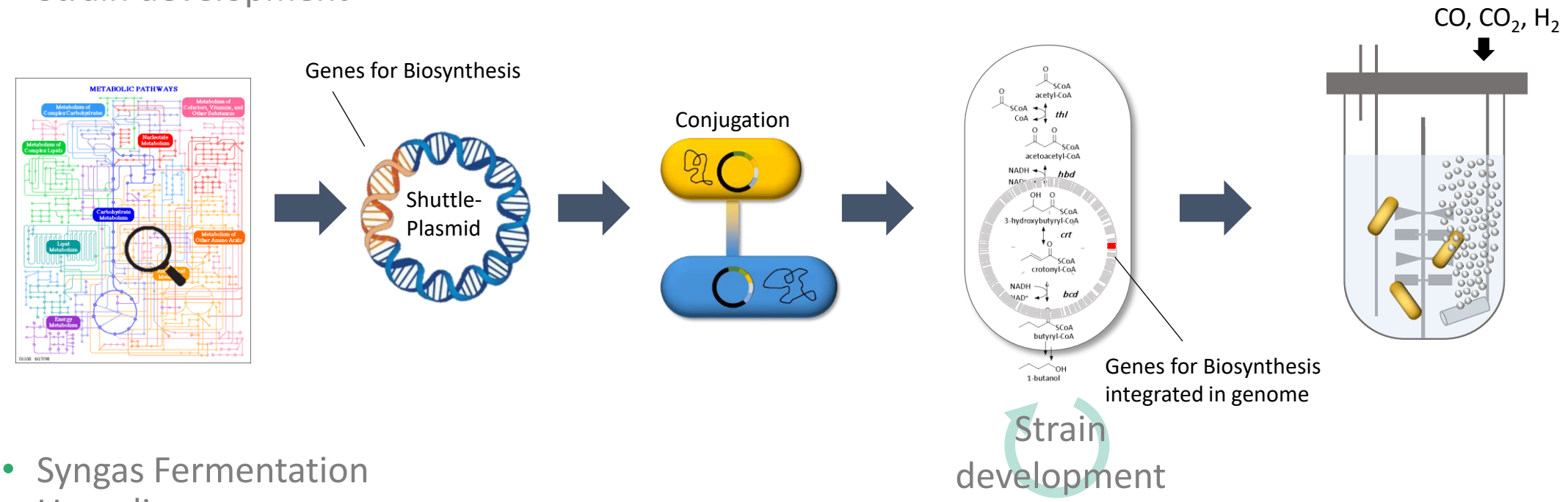
The Aim

- Utilization of Carbon dioxide as carbon source
- Microbial production of butanol and hexanol with high titers
- Use of *Clostridium ljungdahlii* an acetogenic bacterium (genetically accessible)
- Stable expression strain → necessary for continuous fermentation

Strategy: Using Synthetic Biology for Production of Biochemicals

- Pathway selection
- PCR or gene synthesis & cloning in a shuttle plasmid
- Conjugation of the plasmid and genomic integration of the pathway genes
- Strain development

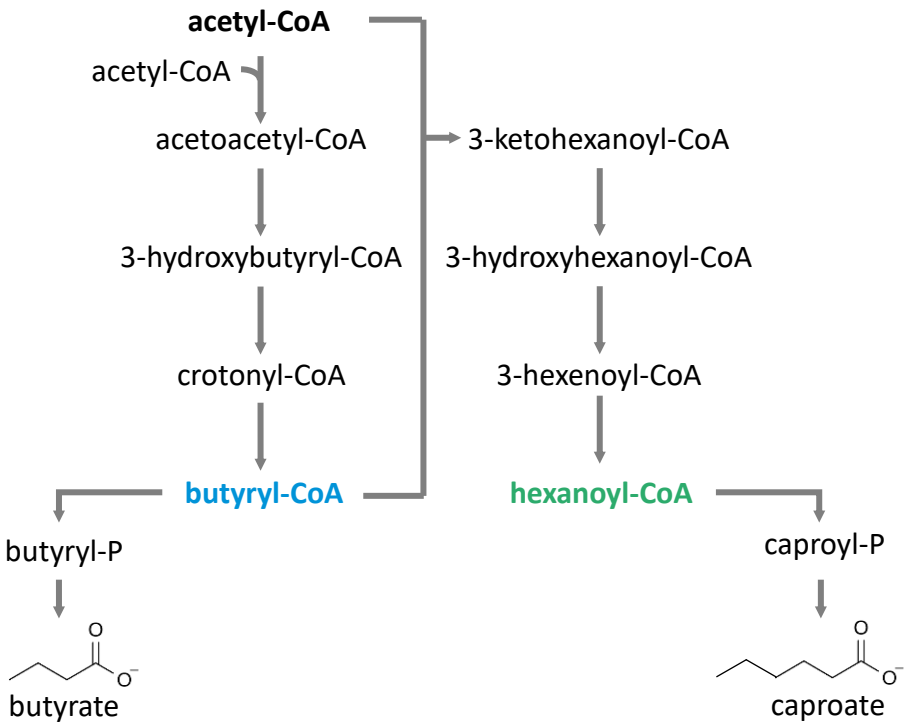


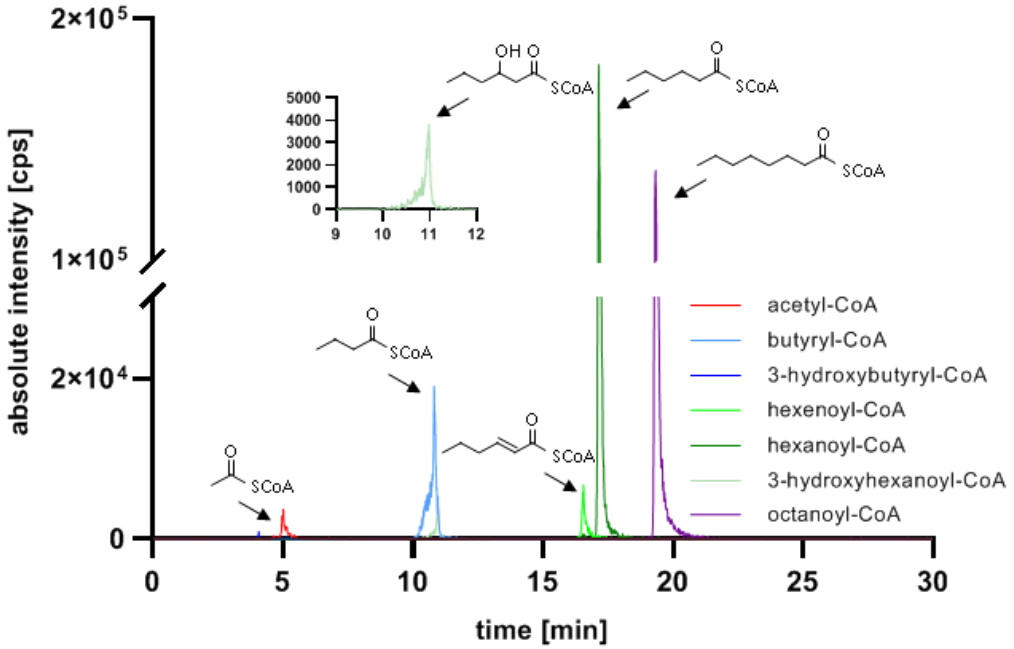
- Syngas Fermentation
- Upscaling
- Downstream processing → purification of the products

Philipps, G., de Vries, S., & Jennewein, S. (2019). Development of a metabolic pathway transfer and genomic integration system for the syngas-fermenting bacterium Clostridium ljungdahlii. *Biotechnology for biofuels*, *12*(1), 1-14.

Pathway selection – Butanol and Hexanol Biosynthesis

- *Clostridium kluyveri* produces butyrate and caproate from acetyl-CoA with butyryl-CoA and hexanoyl-CoA as intermediate

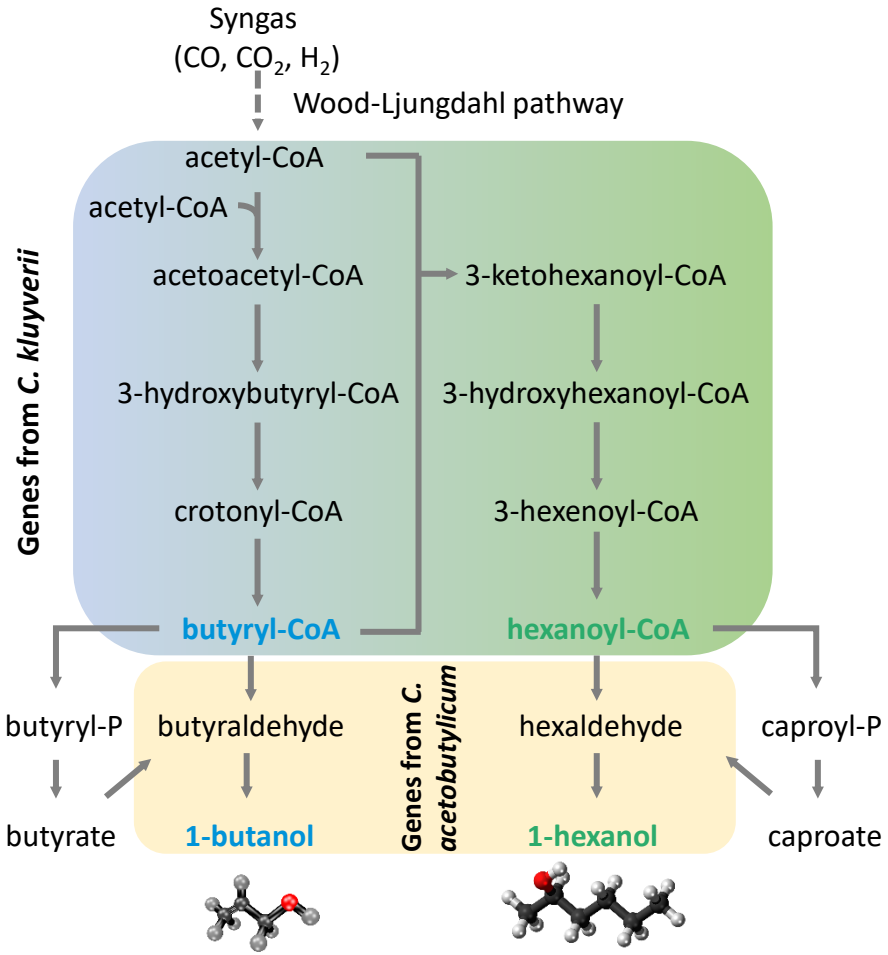


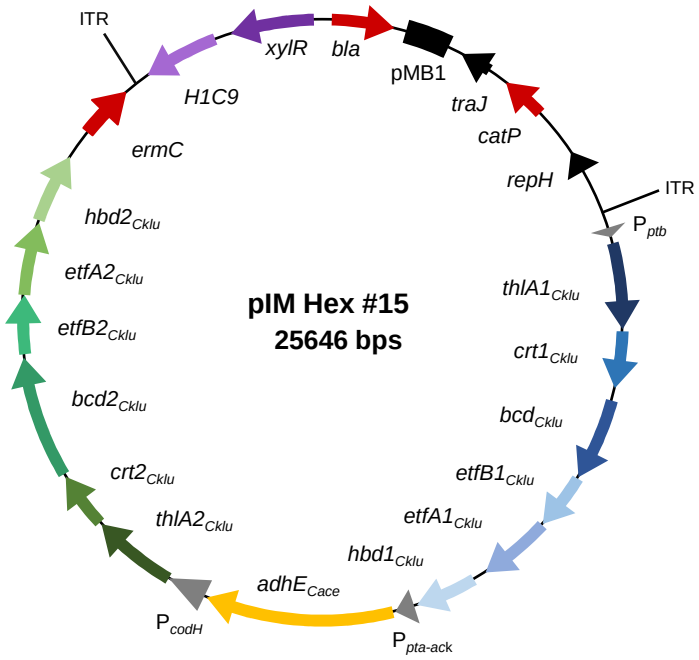


→ *C. kluyveri* genes as a promising basis for butanol & hexanol biosynthesis

Cloning of the Butanol and Hexanol Biosynthesis Genes

- Genes for butyryl-CoA and hexanoyl-CoA biosynthesis were used from *Clostridium kluyveri* and genes for reduction to the alcohols butanol and hexanol from *C. acetobutylicum* → cloned in pIM Hex#15



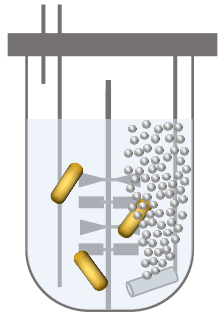


Heterologous production of Butanol and Hexanol

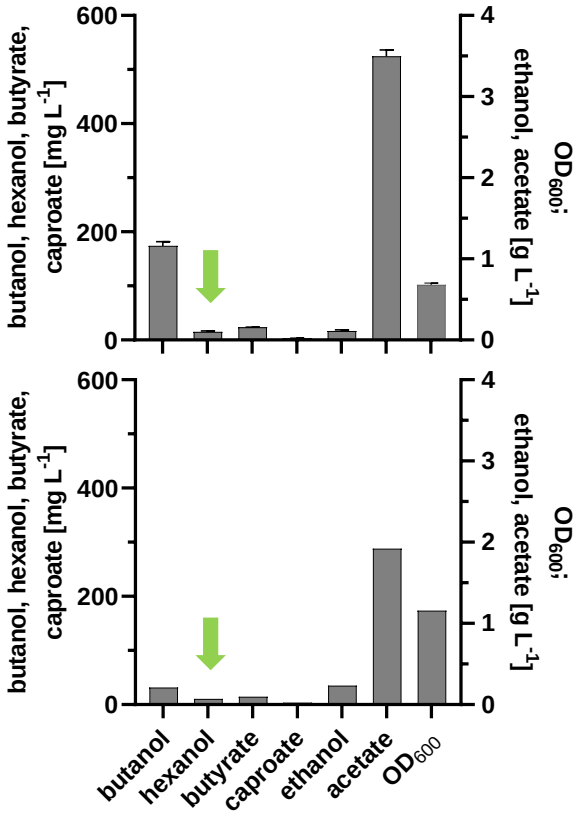
- Conjugation of pIM Hex#15 in *C. ljungdahlii* and genomic integration of the butanol & hexanol biosynthesis gene cluster

Cultivation in serum bottle

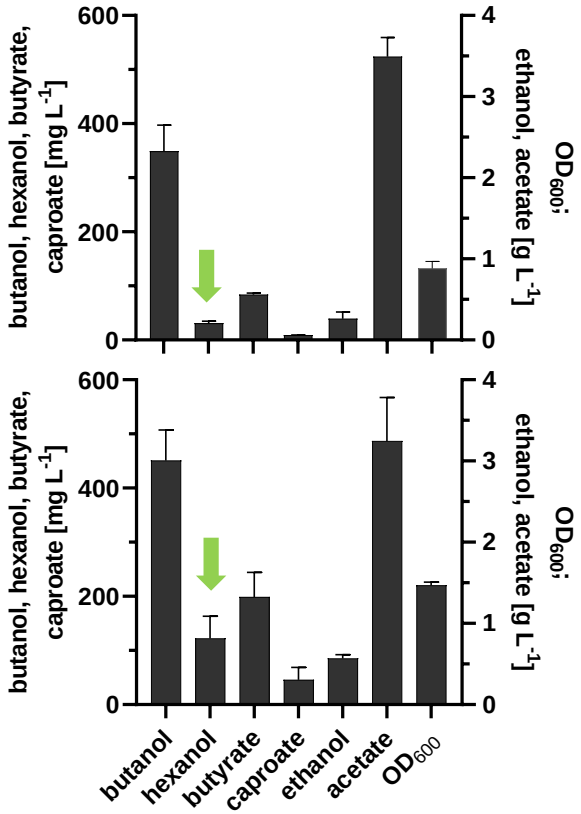
Cultivation in 2-L fermenter



Plasmid-based strain



Genomic integration strain

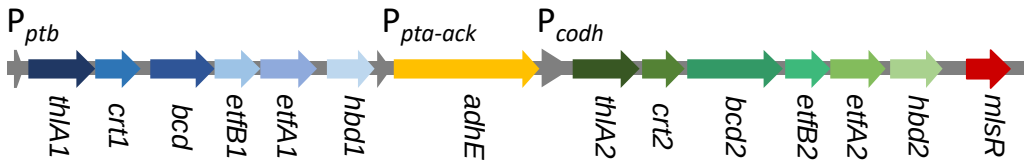


→ Increase in butanol and hexanol titer by genomic integration

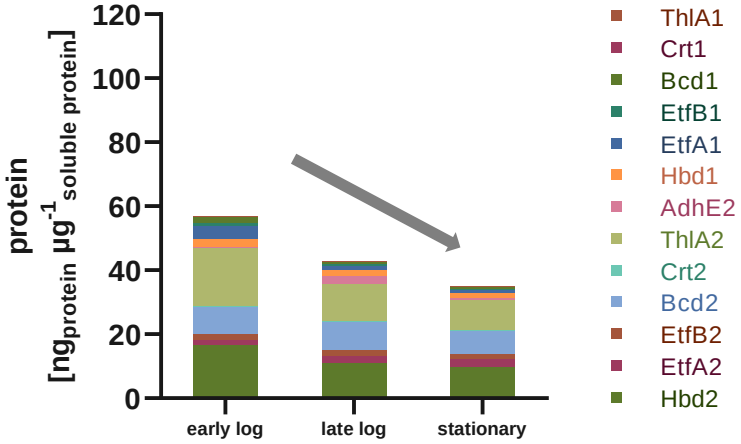
→ Increase in product titers in 2L fermenter scale

Using Targeted Proteomics for identification of putative bottlenecks

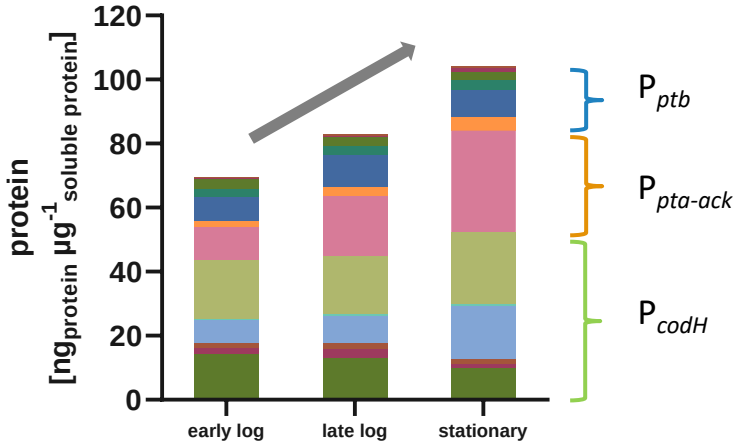
- Targeted proteomics analysis were conducted from 2-L fermentation with transgenic *C. ljungdahlii* strains



Plasmid-based strain



Genomic integration strain



LC-MSMS analysis

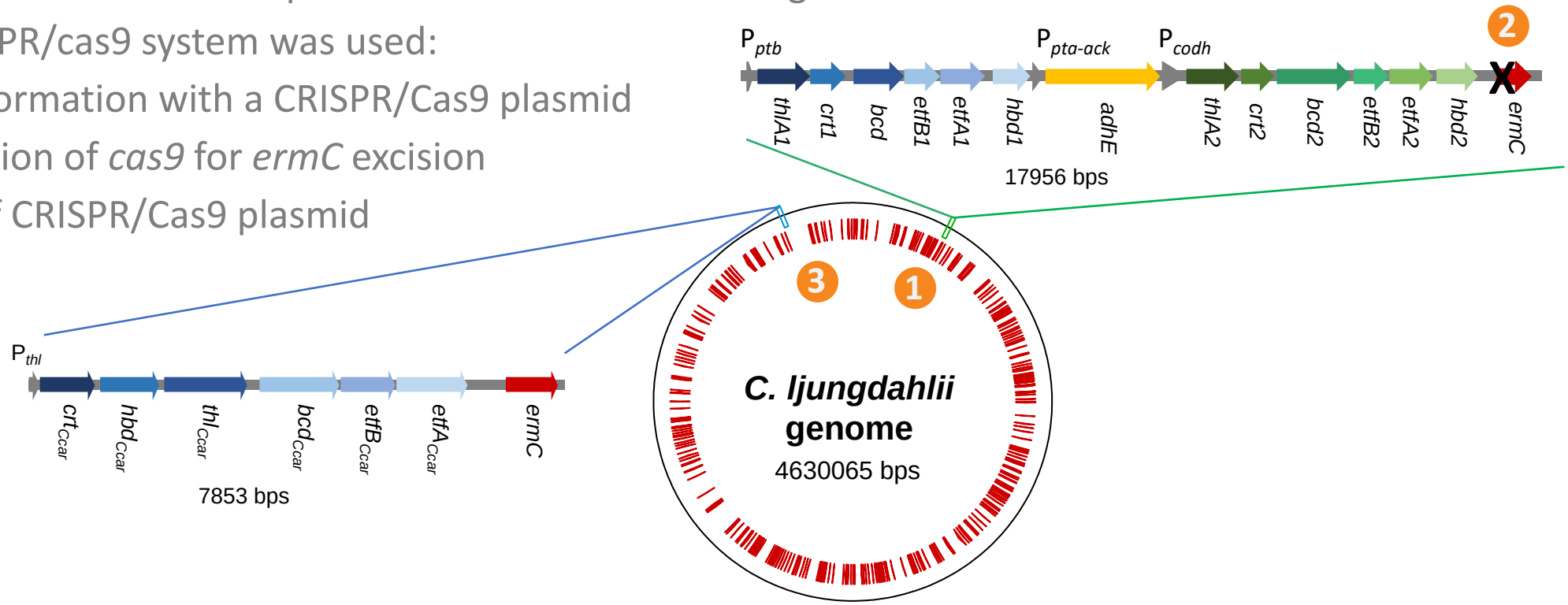
→ Low enzyme expression of cluster under P_{ptb} could be a bottleneck for product formation

Strain development

- Integration of the complete cluster (17956 bps) at position 364,230 in the *C. ljungdahlii* genome 1
- For further strain development the resistance marker gene *ermC* needed to be removed 2

  → CRISPR/cas9 system was used:

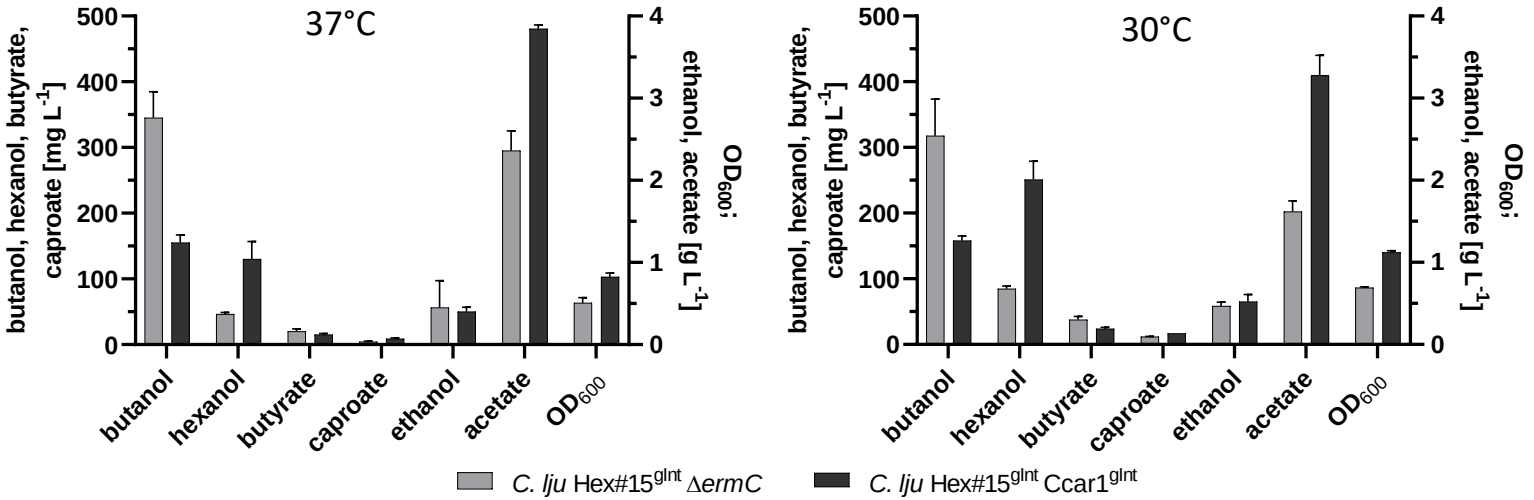
  - transformation with a CRISPR/Cas9 plasmid
  - induction of *cas9* for *ermC* excision
  - loss of CRISPR/Cas9 plasmid



- Conjugation and second integration of a second butanol (& hexanol) biosynthesis cluster from *C. carboxidivorans* at position 4,355,156 in the *C. ljungdahlii* genome 3

Strain development and process development results

- The single genomic integration strain was compared with the double genomic integration strain
- Strain performance was compared at 37°C and 30°C cultivation temperature

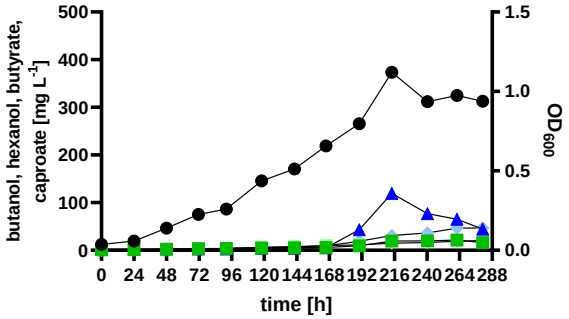


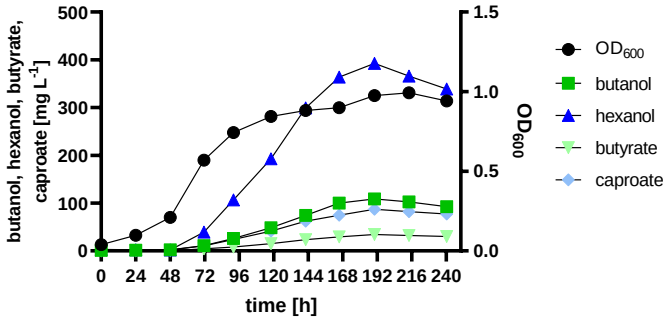
→ The double genomic integration strain showed an increase in hexanol titer at the expense of butanol

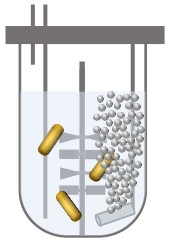
→ A lower incubation temperature improved hexanol titer (→ 251 mg/L)

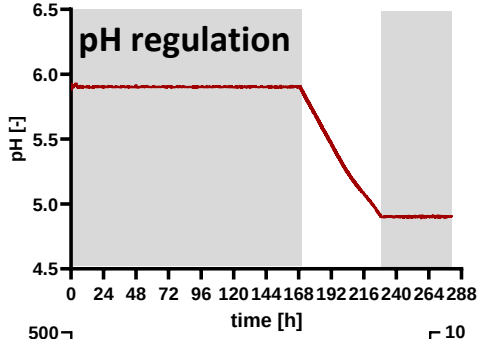
Fermentation and process development results

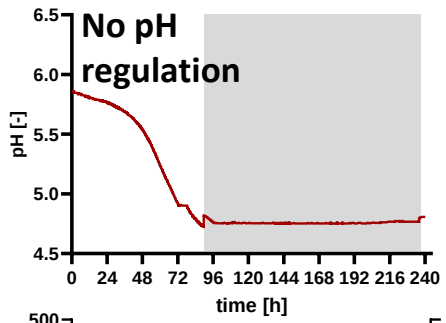
- The double genomic integration strain was grown in a 2-L fermenter at 30°C at different pH profiles

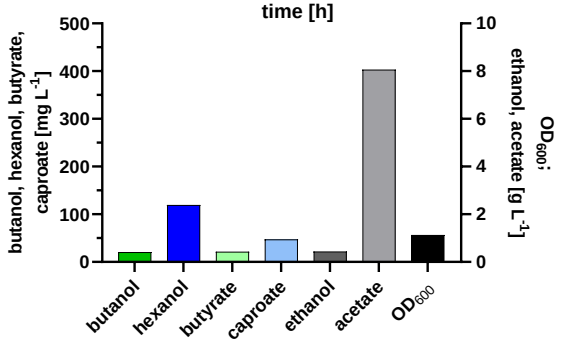


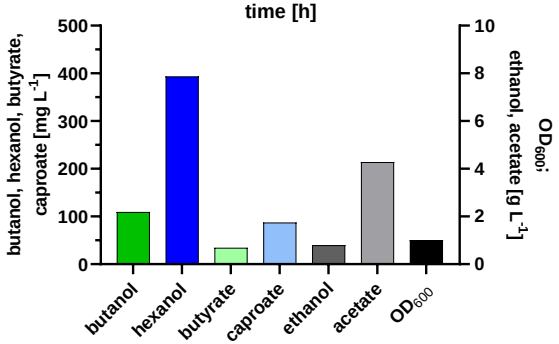












→ Natural acidification of the double genomic integration strain triggered alcohol production

Summary for Metabolic engineering of. *C. ljungdahlii*

- *C. ljungdahlii* wildtype (genetically accessible) → no native butanol and hexanol formation

Metabolic engineering

- Introduction of heterologous gene cluster
- Plasmid-based expression → 174 mg/L butanol, 15 mg/L hexanol
- Genomic integration
- CRISPR/Cas9 for removal of antibiotic resistance gene
- Introduction of additional pathway genes
- Genomic integration

Process development

- Reduced cultivation temperature (30°C)
- Natural acidification until pH 4.75

→ **heterologous** hexanol production with *C. ljungdahlii* → 109 mg/L butanol and 393 mg/L hexanol on 20% CO_2, 80% H_2

Lauer, I., Philipps, G., & Jennewein, S. (2022). Metabolic engineering of *Clostridium ljungdahlii* for the production of hexanol and butanol from CO2 and H2. *Microbial Cell Factories*, *21*(1), 1-18.

Conclusion and Outlook

- A strain which does not naturally produce butanol and hexanol could be engineered as producer strain
- Proof-of-concept for the valorization of CO_2 to C4 & C6 alcohols
- Stable expression was possible without addition of antibiotics
- Product ratio can be altered

Further optimization

- Change of promoter / RBS / genes
- Process parameters and medium composition
- Increase in biomass

Development of CO_2 and CO_2-containing gases fermentation by solvent producing strains

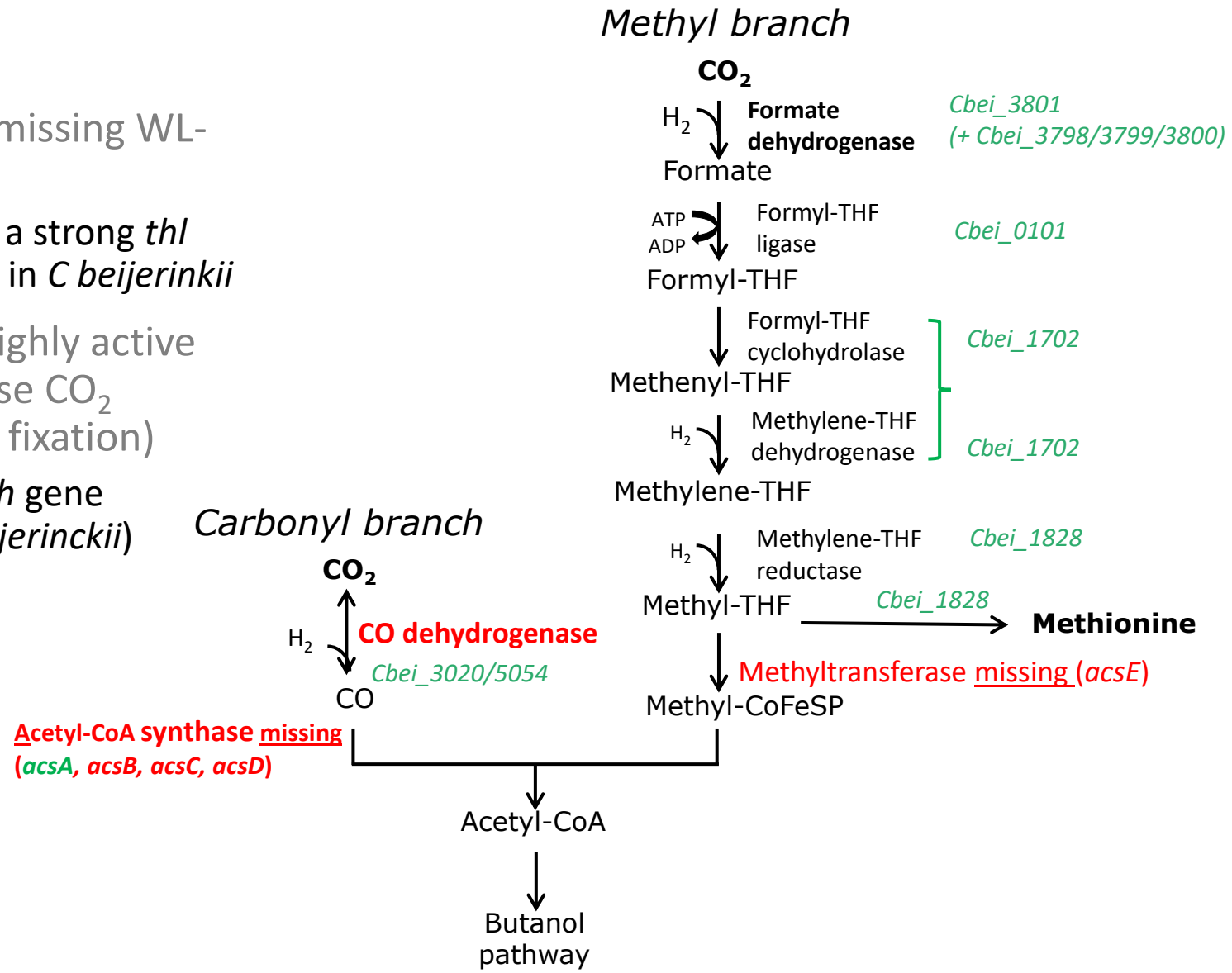
Dr. Ana Lopez-Contreras

Wageningen Food & Biobased Research

Results

1. Construction of expression vectors for missing WL-pathway genes:
   - Vector with ***acsA*** gene under control of a strong *thl* promoter constructed and transformed in *C beijerinkii*
2. Additional strategy: introduction of a highly active formate dehydrogenase gene to increase CO_2 conversion to formate (first step in CO_2 fixation)
   - Vector with highly active *Thiobacillus fdh* gene constructed (codon-optimized for *C. beijerinckii*)

Characterization of mutants ongoing



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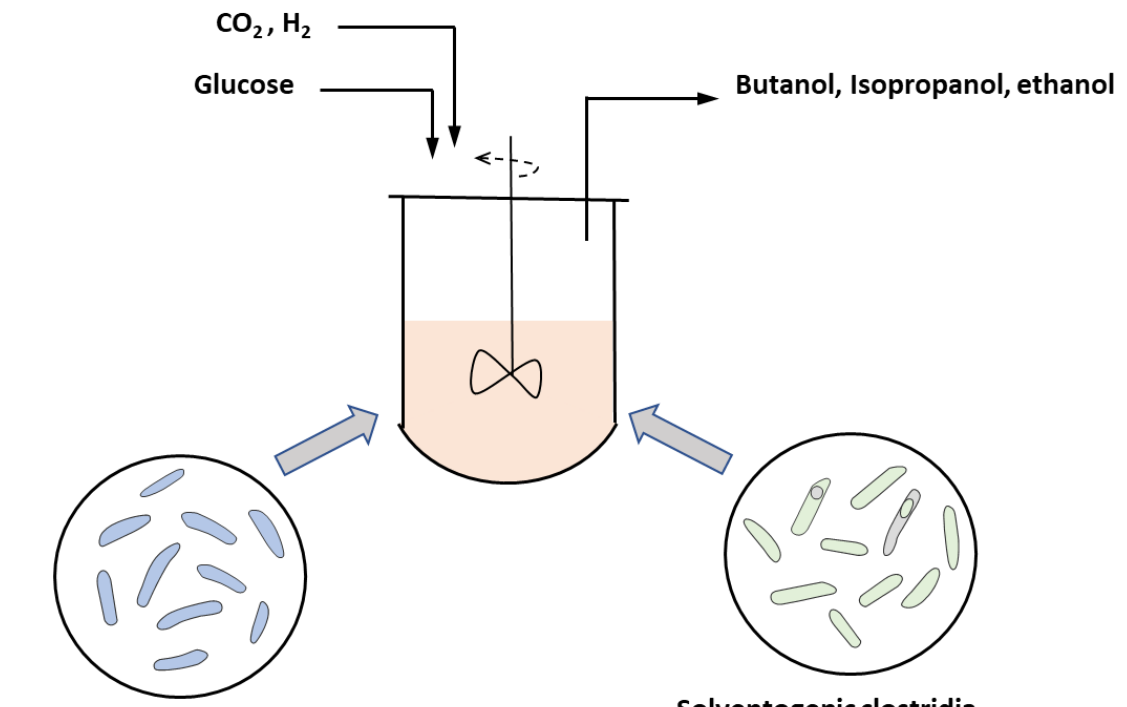
Results: Co-cultures of acetogenic and solventogenic Clostridia

Indirect approach to CO_2 use by solventogens



Gas-fermentation in 2L reactor

- Phase 1: acetogenic bacteria *C. autoethanogenum* or *C. ljungdahlii* on gas mix H_2:CO_2 (80%:20%), no fructose
- Phase 2: at the end of Phase 1 gas fermentation, reactor inoculated with solventogen *C. acetobutylicum* with addition of glucose 10.5 g/L (N_2 gas, no H_2/CO_2)

- Co-cultures demonstrated the feasibility of converting CO_2 to alcohols via cross-feeding from acetogens to solventogens

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Development of CO_2 and CO_2-containing gases fermentation by solvent producing strains: Main achievements so far

- Genes for CO_2 utilisation needed in solventogens are identified
- Transformants obtained: harboring the CODH gene, harboring FDH gene
- Co-cultures acetogen-solventogen tested: acetate from CO_2 is utilised by solventogens for production of acetone, butanol and ethanol
- Tolerance of solventogens to raw gases: Benzene, toluene and xylene (BTX) at high concentrations show no toxicity on the cultures

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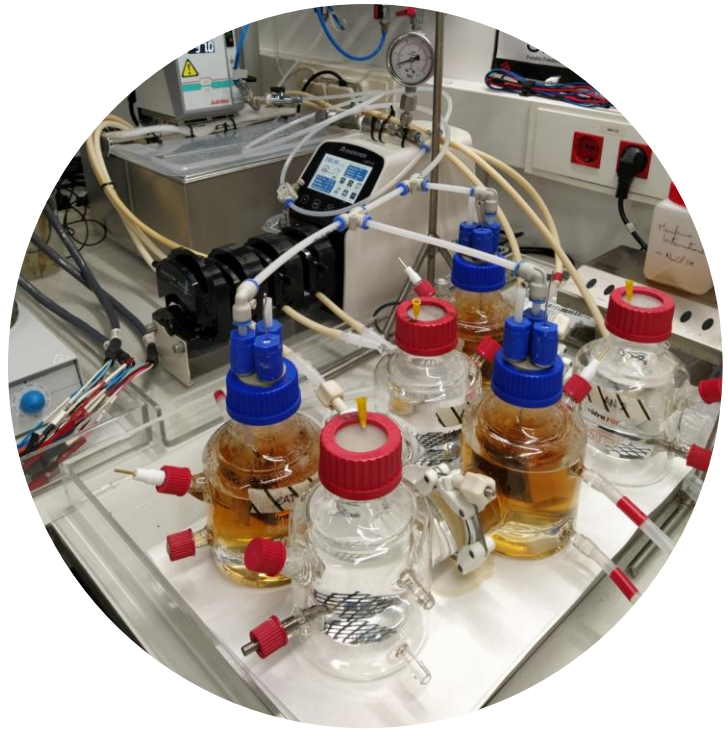
Enhancing butanol production by *Clostridium beijerinckii* through cathodic electro-fermentation approach



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The **BIOCON-CO2** project receives funding from the EU Horizon 2020 Research and Innovation programme, under G.A. No 761042.

Daniele Molognoni,[1] Ana Lopez Contreras[2], Montse Bosch[1], Pau Bosch-Jimenez[1], Eduard Borràs[1]

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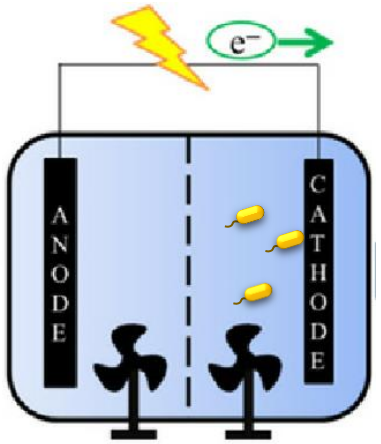
[2] Wageningen Food & Biobased Research, 6700 AA Wageningen (The Netherlands)

ABE electro-fermentation by *Clostridium beijerinckii*

- Pure strain of *Clostridium beijerinckii* was selected for EF proof of concept
- Gram-positive, strictly anaerobic bacteria, able to ferment glucose
- Biphasic fermentation: acid and solvent fermentation phase
- Alcohols are toxic for bacterial growth → need of product recovery

Electrofermentation (EF) is an electrochemically influenced, spontaneous fermentation, i.e. an electrochemical process to control fermentation pathways using current (Rabaey & Rozendal, 2010).

Rabaey K, Rozendal RA. Microbial electrosynthesis - revisiting the electrical route for microbial production. Nature Reviews Microbiology 2010;8:706–16.

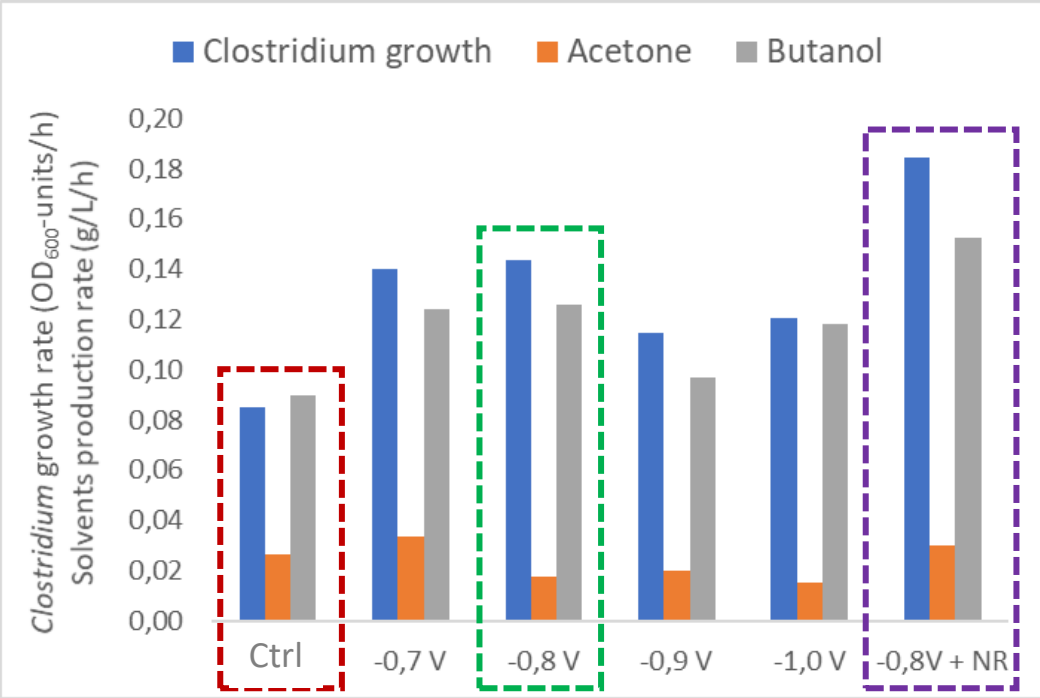


Supplying e^- by a cathode → raised NADH/NAD^+ ratio → increase of butanol production, yield and proportion in the fermentation broth

Highlights of EF:

- Varying redox potential of fermentation broth
- Manipulating intracellular **NADH/NAD^+ ratio**
- **Cathodic EF** → *in-situ* H_2 production (additional reducing power)

Effects of EF approach on ABE process

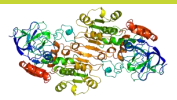


- Applied cathode potential at -0.8 V vs Ag/AgCl resulted in optimal scenario
- *Clostridium* growth rate increased of 56% vs control
- Butanol production increased of 44% vs control
- Decrease of fermentation time
- Higher process selectivity towards butanol

Addition of neutral red (NR) as redox mediator:

- 0,5 mM neutral red addition (exogenous redox mediator) further increased *Clostridium* growth rate of 28%
- Butanol production further increased of 15%
- However, process selectivity and current density decreased

Parameter	Ctrl	E_{cat} -0,7 V	E_{cat} -0,8 V	E_{cat} -0,9 V	E_{cat} -1,0 V	E_{cat} -0,8 V + 0,5 mM NR
Clostridium growth rate (1/h)	0,09	0,14	0,14	0,11	0,12	0,18
acetone prod. rate (g/L/h)	0,03	0,03	0,02	0,02	0,02	0,03
butanol prod. rate (g/L/h)	0,09	0,12	0,13	0,10	0,12	0,15
butanol selectivity (%)	78%	78%	87%	82%	88%	82%
current density (A/m^2)	-	0,4	2,1	11,2	35,8	0,9
EF coefficient (%)	-	0,1%	0,4%	3%	7%	0,2%



BIOCON-CO_2

Enzymatic production of formic acid from CO_2

Tom Ewing, Daan van Vliet, Lorenzo Schwerdtfeger, Guus Frissen, Rick van der Vondervoort, Mattijs Julsing & Carmen Boeriu

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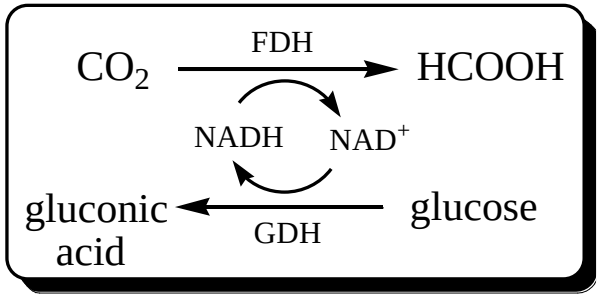
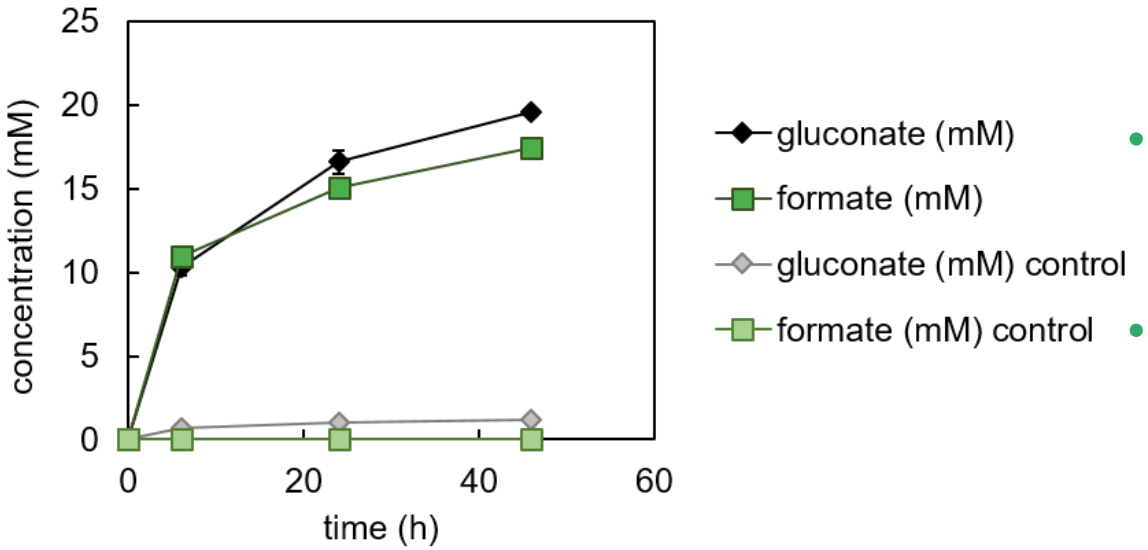
Enzymatic production of formic acid from CO_2

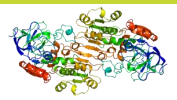


- Formic acid is a C1 carboxylic acid with applications in leather processing, animal feed and as a chemical building block
- Formic acid can be selectively produced from CO_2 under mild reaction conditions by reduction using the enzyme formate dehydrogenase (FDH)
- FDH uses NADH as a redox cofactor, and this expensive cofactor must be regenerated to enable a cost-effective process. This can be achieved by the oxidation of glucose using an NAD^+-dependent glucose dehydrogenase (GDH)

Enzymatic production of formic acid from CO_2



- an enzymatic system for formate production was designed, based on an FDH from *Thiobacillus* sp. (Ts_FDH) with increased CO_2-reducing activity compared to other FDHs (*Choe et al. , PLOS One, 2014*)
- In combination with GDH, Ts-FDH was used to produce 0.8 g/L (17 mM) formate from CO_2 in 46 h
- Formate was also produced from gas mixtures mimicking steel industry off-gasses, titres were lower (~4 mM), likely due to lower CO_2 content and inhibition by CO and/or H_2
- In future, formate titres and production rate must be increased, e.g. by using optimised engineered enzymes



BIOCON-CO_2

CO_2 Valorisation: Multienzymatic synthesis of lactic acid

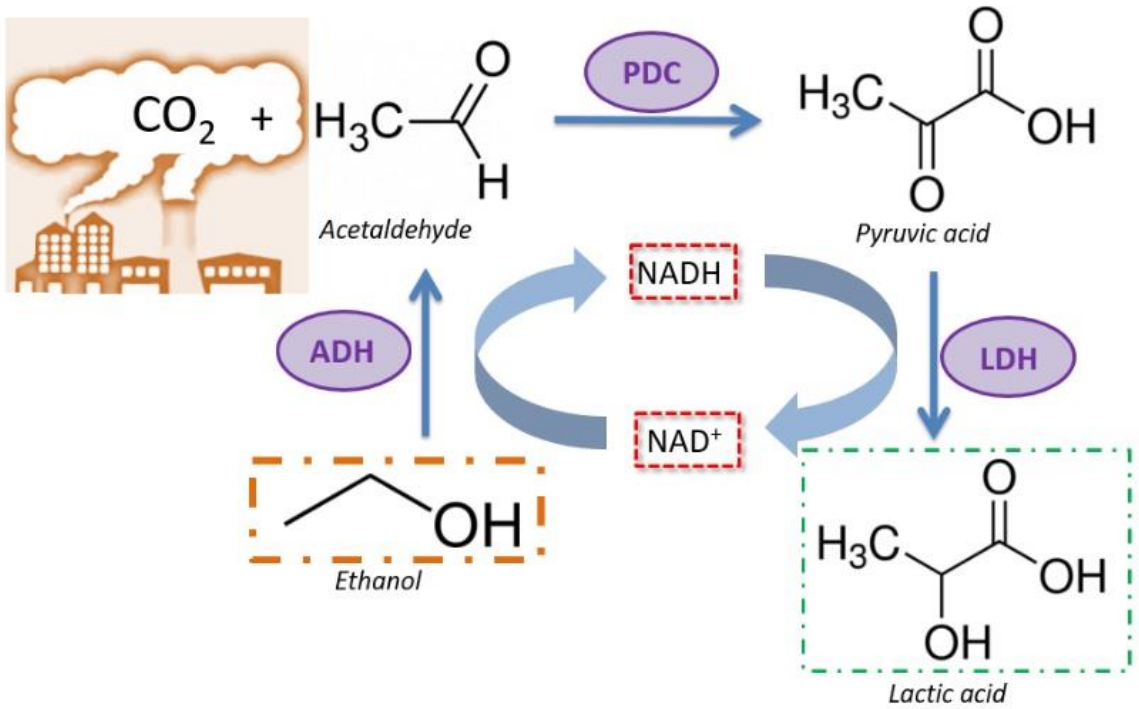
Albert Carceller Lladó

Department of chemical, biological and environmental engineering

Applied biocatalysis and bioprocess engineering

UAB
Universitat Autònoma
de Barcelona

Multienzymatic synthesis of lactic acid from CO_2



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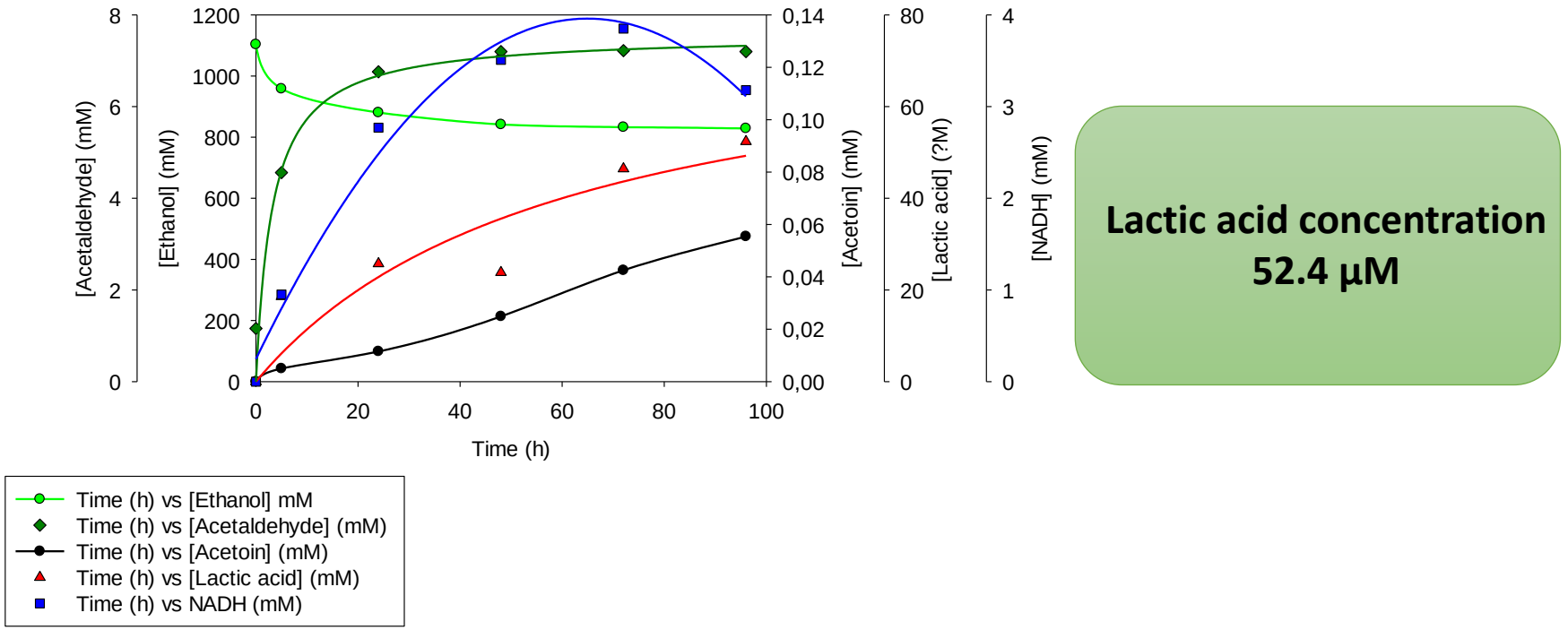
Reaction using synthetic gases mimicking real off-gases composition

- Iron&steel industry off-gas composition (Blast Furnace)

  1.2% O_2, 3.8% H_2, 23.9% CO, **24.5% CO_2**, 46.6% N_2



Lactic acid concentration 52.4 µM

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Thank you

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