KTH ROYAL INSTITUTE
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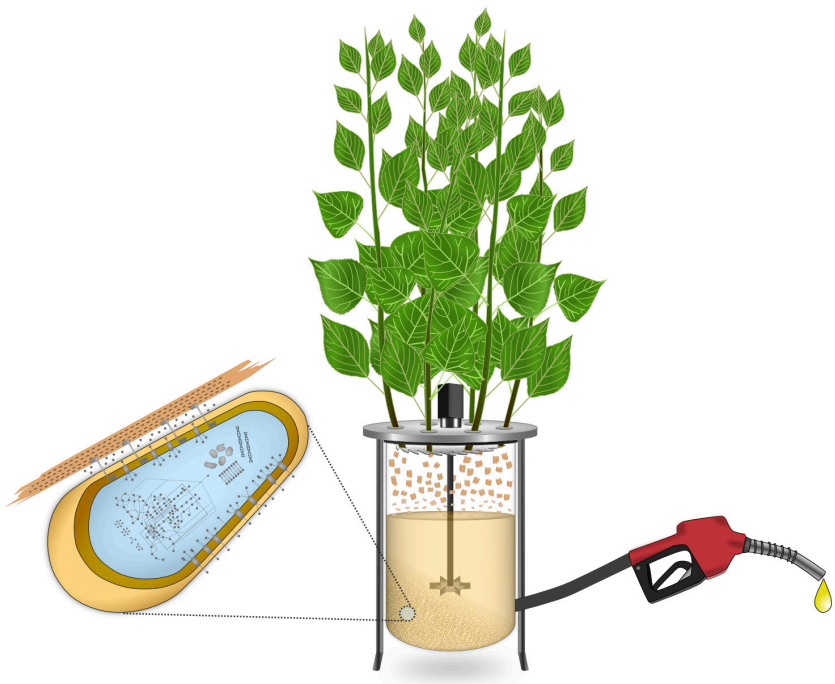
Doctoral Thesis in Biotechnology

Insights into the metabolism of *Clostridium thermocellum* for cellulosic ethanol production

JOHANNES YAYO



Stockholm, Sweden 2022

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JOHANNES YAYO

Academic Dissertation which, with due permission of the KTH Royal Institute of Technology, is submitted for public defence for the Degree of Doctor of Philosophy on Tuesday the 8th of November 2022, at 9:00 a.m. in Kollegiesalen, Brinellvägen 8, Stockholm.

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To my parents

Abstract

The societal goal of reaching net-zero CO_2 emissions requires development of integrated biorefineries to produce biomass-derived fuels and chemicals. For sustainable second-generation bioethanol production, consolidated bioprocessing with the thermophile *Clostridium thermocellum* is regarded as a promising concept in view of the microorganism's native ability to efficiently degrade plant cell wall material. However, for industrial implementation, improvements in ethanol titer and yield are needed. The aim of this thesis was to increase knowledge on the metabolism of *C. thermocellum* and thereby guide future metabolic engineering strategies to maximize the ethanol titer and yield.

Yield improvements and fundamental studies into the metabolism of *C. thermocellum* would benefit from higher utilization of hexose monomers as well as minimized byproduct formation. To investigate underlying mechanisms for growth on glucose and fructose, laboratory evolution in chemostats together with genome sequence-based reverse engineering were applied. This successfully revealed two underlying mutations with (regulatory) roles in metabolism or transport of the monosaccharides. Together, these mutations enable reproducible and constitutive growth and are relevant for follow-up studies into transport and upper glycolysis. Separately, the mechanism behind the surprising byproduct formation of secreted amino acids was investigated by knock-out studies in NADPH-supplying and -consuming pathways. Physiological characterization in cellobiose- or ammonium-limited chemostats of mutant strains, which had deletions in the NADPH-forming malate shunt or in the putatively ferredoxin-dependent ammonium assimilation, demonstrated a central role of NADPH in driving amino acid secretion. The findings indicated that electron availability will be crucial for further yield improvements in the NADH-dependent ethanol pathway.

Fundamental mechanisms that might contribute to improved ethanol titer were addressed by studying thermodynamic and biophysical limitations. The pyrophosphate (PP_i)-dependent glycolysis of *C. thermocellum* has been hypothesized to increase the overall ATP yield at the expense of the overall driving force. Knock-out studies combined with functional annotation of potential PP_i-sources questioned this trade-off and increased

knowledge of the PP_i metabolism. The chaotropic effect (biophysical toxicity) of ethanol is commonly counteracted by lowering the cultivation temperature. Here, physiological characterization at varying ethanol titers demonstrated improved growth and fermentation at lower temperature. Comparisons to a non-ethanol producing mutant indicated both thermodynamic and biophysical limitations specifically in the ethanol pathway.

Overall, these findings suggest that improvements in ethanol titer and yield would benefit from a simplified glycolysis that is engineered for a high driving force. While this work is beneficial for second-generation ethanol production, these findings can also be broadly applicable in the research and development of *C. thermocellum* as a cell factory for sustainable production of other fuels and chemicals.

Keywords

Clostridium thermocellum, ethanol, glucose, fructose, amino acids, pyrophosphate, chaotropicity, thermodynamic driving force, laboratory evolution, chemostats, metabolic engineering

Sammanfattning

Samhällsmålet att nå nettonoll CO_2 utsläpp kräver en utveckling av integrerade bioraffinaderier för att producera bränslen och kemikalier baserade på biomassa. För hållbar andra-generationens bioetanol-produktion betraktas konsoliderad bioprocessering med termofilen *Clostridium thermocellum* som ett lovande koncept baserat på dess naturliga förmåga att effektivt bryta ner växtcellväggar. Emellertid krävs ökad titer och utbyte av etanol för att nå industriell implementering. Målet med denna avhandling var att öka kunskapen om *C. thermocellums* metabolism och därmed vägleda framtida strategier för att maximera titern och utbytet av etanol genom metabolic engineering.

Förbättringar i utbytet samt fundamentala studier på metabolism hos *C. thermocellum* skulle gynnas av ett större utnyttjande av C_6-mono-sackarider samt minskad produktion av biprodukter. Underliggande mekanismer för tillväxt på glukos och fruktos undersöktes med laboratory evolution i kemostater samt genomsekvensbaserad reverse engineering. I denna studie avslöjades två underliggande mutationer med (regulatoriska) roller i metabolismen eller transporten av dessa monosackarider. Tillsammans möjliggjorde dessa mutationer reproducerbar och konstitutiv tillväxt. Mutationerna är även relevanta för uppföljningsstudier av sockertransport och den övre glykolysen. Därutöver studerades den oväntade biproduktgruppen, aminosyror, genom knockoutstudier på NADPH-producerande och -konsumerande reaktionsvägar. Stammar med knockouts i den NADPH-producerande malatshunten eller i den potentiellt ferredoxinkopplade ammoniumassimileringen karaktäriserades fysiologiskt i cellobios- eller ammoniumbegränsande kemostater. Detta visade att NADPH har en central roll i att driva aminosyrautsöndring. Dessa upptäckter indikerade att elektrontillgängligheten är kritisk för att öka utbytet i den NADH-baserade etanolproduktionen.

Fundamentala mekanismer som skulle kunna bidra till förbättrad titer av etanol studerades från termodynamiska och biofysiska perspektiv. En rådande hypotes har varit att den pyrofosfat (PP_i)-beroende glykolysen hos *C. thermocellum* ökar ATP-utbytet på bekostnad av den totala termo-dynamiska drivkraften. Knockoutstudier kombinerat med funktionell annotering av potentiella PP_i-källor ifrågasatte denna hypotes och ökade

förståelsen av PP_i metabolismen. Den kaotropiska effekten (biofysisk toxicitet) av etanol dämpas ofta i industriella processer genom att sänka odlingstemperaturen. Här demonstrerade fysiologisk karaktärisering vid olika etanoltiter att tillväxt och fermentering förbättras vid lägre temperaturer. En jämförelse mellan en modifierad icke-etanolproducerande stam och vildtypen indikerade att etanolproduktionen är begränsad av både termodynamiska och biofysiska faktorer.

I helhet antyder dessa forskningsresultat att förbättringar i titern och utbytet av etanol skulle gynnas av en förenklad glykolys, konstruerad för att ge en hög termodynamisk drivkraft. Fastän denna avhandling fokuserar på andra-generationens etanolproduktion kan dessa forskningsrön även appliceras mer brett i forskning och utveckling av *C. thermocellum* som en cellfabrik för hållbar produktion av andra bränslen och kemikalier.

Public defense of dissertation

This thesis will be defended on Tuesday the 8th of November 2022, at 9:00 a.m. in Kollegiesalen, Brinellvägen 8, Stockholm.

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List of appended papers

Paper I
Laboratory Evolution and Reverse Engineering of *Clostridium thermocellum* for Growth on Glucose and Fructose.
Yayo, J., Kuil, T., Olson, D.G., Lynd, L.R., Holwerda, E.K., and van Maris, A.J.A.
Appl. Environ. Microbiol., 2021, 87(9), e03017-20.

Paper II
The role of redox-cofactor regeneration and ammonium assimilation in secretion of amino acids as byproducts of *Clostridium thermocellum*.
Yayo, J., Rydzak, T., Kuil, T., Karlsson, A., Harding, D.J., Guss, A.M., and van Maris, A.J.A.
Submitted for publication.

Paper III
Functional analysis of H^+-pumping membrane-bound pyrophosphatase, ADP-glucose synthase, and pyruvate phosphate dikinase as pyrophosphate sources in *Clostridium thermocellum*.
Kuil, T.*, Hon, S.*, Yayo, J., Foster, C., Ravagnan, G., Maranas, C.D., Lynd, L.R., Olson, D.G., and van Maris, A.J.A.
Appl. Environ. Microbiol., 2021, 88(4), e01857-21.
*Equal contribution

Paper IV
Ethanol tolerance of *Clostridium thermocellum*: the role of chaotropicity, temperature and pathway thermodynamics on growth and fermentative capacity.
Kuil, T., Yayo, J., Pechan, J., Küchler, J., and van Maris, A.J.A.
Submitted for publication.

Contribution to appended papers

Paper I
First author. Designed and performed the majority of the experiments, wrote the first version of the manuscript, and revised the manuscript together with the co-authors.

Paper II
First author. Designed and performed the majority of the experiments, wrote the first version of the manuscript, and revised the manuscript together with the co-authors.

Paper III
Second author. Contributed to the design of experiments, experimental work, and revised the manuscript together with the co-authors.

Paper IV
Second author. Contributed to the design of experiments, experimental work, and revised the manuscript together with the co-authors.

Other scientific contributions

Laboratory evolution and reverse engineering of *Clostridium thermocellum* for growth on glucose and fructose, Metabolic Engineering 14 Conference, 11-15th of July, 2021, *Poster Presentation*.

Insights into hexose sugar and PP_i metabolism of *Clostridium thermocellum* for improved cellulosic ethanol production, 44th Symposium on Biomaterials, Fuels and Chemicals, 1-4th of May, 2022, New Orleans, Louisiana, USA, *Oral and Poster presentation*.

A novel bacterial GT2 polysaccharide synthase can produce mixed-linkage (1,3;1,4)-β-glucan. Chang S-C, Kao M., Karmakar R., Diaz S., Xing X, Yayo J., Vilaplana F., Abbott W., Hsieh Y.S.Y., *Submitted for publication*. Contributed to the design of the anaerobic cultivations and the experimental work to cultivate the bacteria.

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1. Introduction

1.1 Towards net-zero emissions

The technological and economic advancements in the last centuries have improved health, welfare, services, and production and quality of goods. With these developments, the demand on energy has rapidly increased and is expected to continue to grow in this century (1). The usage of energy in the past has been the largest contributor to greenhouse gas (GHG) emissions, followed by non-energy related industrial process emissions and land-use (e.g. deforestation and agricultural emissions from soil and livestock) (2). Earth system models conclude that these emissions must reach net zero in order to stabilize the global-mean temperature (3). In order to limit the increase in temperature to below 2 °C above pre-industrial levels, predicted future scenarios show a need to achieve net-zero CO_2 emissions around the middle of this century, with net negative CO_2 emissions thereafter (4). The International Energy Agency (IEA) recently produced a roadmap in 2020 for a 'Net-Zero Emissions by 2050' scenario based on the most cost-effective, socially accepted and technically feasible solutions including expected economy and population growth (5). The scenario shows a need for CO_2 reductions in all sectors (Figure 1). These reductions are achieved by utilizing a mixture of technologies and measures, including increased efficiencies for buildings, industry and transport, electrification, the use of renewable energy sources, and carbon capture, utilization and storage (CCUS) (Figure 2) (5). For developing countries, these mitigation strategies will likely need to be adapted to allow the required energy demand for economic growth and structural changes for sustainable development while decarbonizing (6).

Reaching net-zero CO_2 emissions in certain sectors will likely be easier than in others. Decarbonizing light-duty transport as well as residential and industrial heating and cooling can be achieved readily by electrification with energy provided by renewable energy sources (e.g. solar and wind) and on-demand non-renewable sources (e.g. nuclear

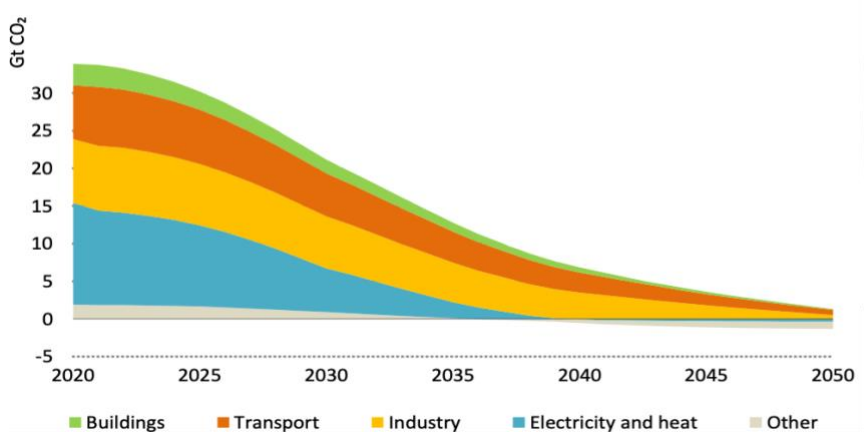


Figure 1. CO_2 reductions by sector in order to reach Net Zero Emissions by 2050. Source: International Energy Agency (2021), *Net Zero by 2050*, IEA, Paris; Net Zero by 2050 scenario – Data product – IEA (https://www.iea.org/data-and-statistics/data-product/net-zero-by-2050-scenario). License: Creative Commons Attribution CC BY-NC-SA 3.0 IGO.

energy and fossil fuels with carbon capture and storage). Other uses of energy are likely to be more difficult to decarbonize, such as production of carbon-intensive structural materials (e.g. steel and cement), back-up electricity generation and/or storage that balance occasional but substantial surges in electricity demands (i.e. replacement of today's natural gas-fired generators), and heavy-duty transport (aviation, shipping, heavy-duty trucks) (5, 7, 8). Being essential to modern society, these sectors are responsible for a quarter of the global CO_2 emissions and are predicted to grow (7). Aviation, shipping and heavy-duty trucks together corresponded to 10% of the global CO_2 emissions in 2019 and calls for new technological solutions to achieve CO_2 neutrality efficiently (8).

1.2 Technologies for the long-distance transport sector

The challenge for long-distance transport by heavy-duty trucks, ships and airplanes resides in maximizing cargo space and payload capacity (7). Hence, high volumetric and gravimetric energy densities are required. This is a disadvantage of closed-cycle batteries, which have a magnitude lower energy density compared to hydrocarbons. Another disadvantage is that

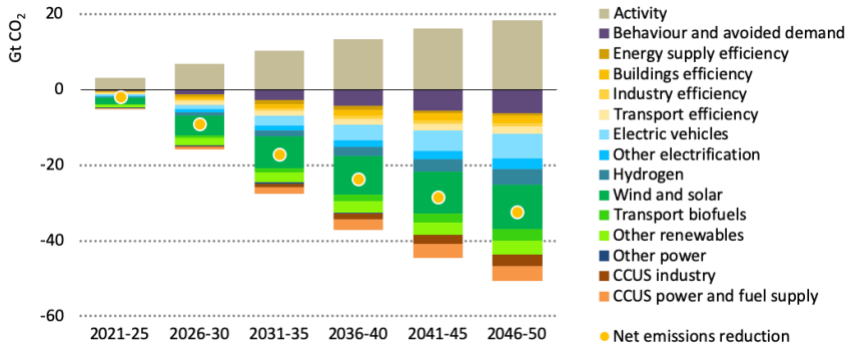


Figure 2. The required expansion in technologies and measures necessary for reaching Net Zero Emissions by 2050 alongside an increasing demand in energy services (annotated as activity) due to global economic and population growth. Source: International Energy Agency (2021), *Net Zero by 2050*, IEA, Paris; Net Zero by 2050 scenario – Data product – IEA (https://www.iea.org/data-and-statistics/data-product/net-zero-by-2050-scenario). License: Creative Commons Attribution CC BY-NC-SA 3.0 IGO. Abbreviations: CCUS, carbon capture, utilization and storage.

batteries retain both reactants and products, whereas fuels are vented upon oxidation and results in lower weight, thus energy consumption, as the combustion progresses (7). Technologies utilizing low-emission fuels, including hydrogen, ammonia, biofuels, synthetic fuels (synfuels), and biomethane, provide promising solutions to this challenge. In net-zero emissions scenarios, shipping and heavy-duty road transport are predicted to use a mixture of technologies based on above mentioned fuels, whereas aviation is fueled by biofuels or synfuels (Figure 3) (5, 7). The choice of fuel will depend on its properties, technology requirements, price, primary energy consumption and its role in the global economy.

1.2.1 Hydrogen

Hydrogen-based fuels, including hydrogen, ammonia, and synfuels, are predicted to play a significant role, especially in the heavy industry and for electricity generation, but also in parts of the transport sector (Figure 3C) (5, 9). Electricity from intermittent energy sources, such as wind and solar power, can be used to produce hydrogen *via* electrolysis (8). The

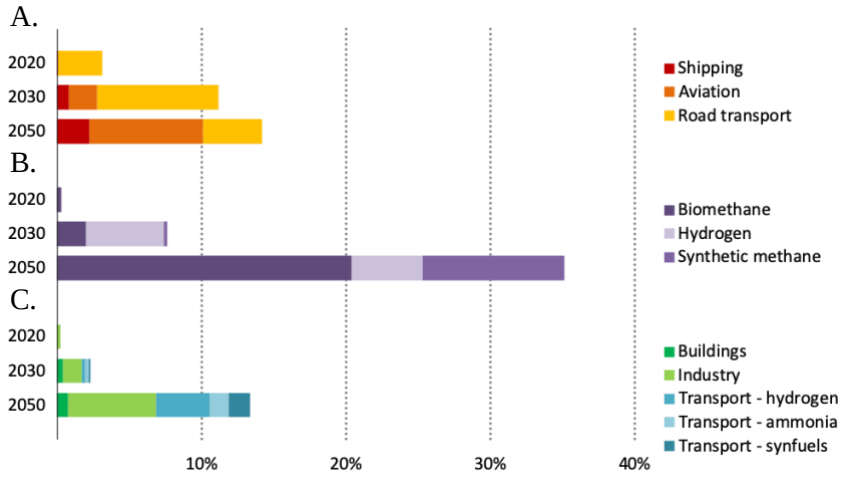


Figure 3. Percentage of global supply of low-emission fuels per sector today and in a Net-Zero Emissions by 2050 scenario (5). (A) The supply of liquid biofuels in the transport sector. (B) The percentage of biomethane, hydrogen, and synthetic methane mixed with natural gas in gas networks. (C) The supply of hydrogen-based fuels, including hydrogen, ammonia, and synthetic fuels (synfuels), in the total final energy consumption (TFC), comprising the industry, transport, buildings, agriculture, and others sectors. Source: International Energy Agency (2021), *Net Zero by 2050*, IEA, Paris; Net Zero by 2050 scenario – Data product – IEA (https://www.iea.org/data-and-statistics/data-product/net-zero-by-2050-scenario). License: Creative Commons Attribution CC BY-NC-SA 3.0 IGO.

hydrogen can be upgraded to ammonia or synfuels (described below). Power can then be generated by combustion or producing electricity using hydrogen fuel cells (9). Direct combustion of hydrogen and ammonia has several benefits, such as low air pollution with water and N_2 as final products, and potentially zero CO_2 emissions if the whole value chain is based on renewables. Combustion of ammonia can however release NO_x gases and therefore requires careful regulation of the thermolysis (7). For liquid hydrogen, the energy release per weight is up to three times higher than long-chain hydrocarbons and thereby reduces operating costs (7). However, due to its low volumetric energy density, even in a pressurized cryogenic state, four times higher volume is required to yield the same amount of energy, necessitating the need for larger storage systems on

airplanes (10). For light- and heavy-duty vehicles, higher energy efficiency than combustion engines can be archived by using a hydrogen fuel cell or a hybrid hydrogen-battery system (7). However, these technologies for airplanes and road vehicles are emerging, currently not deployed in a large scale and ultimately requires development of hydrogen infrastructure, electrolyzer manufacturing capacity, and redesigning aircraft, light-, and heavy-duty vehicles, making these technologies unlikely in the near future (7, 10). Until then, renewable hydrogen can be blended with natural gas in gas networks and used in industries, refineries, and power plants, to reduce CO_2 emissions (Figure 3B and C) (8). For instance, companies in Sweden have recently invested in two large projects aiming to either build new hydrogen-powered steel production or replacing fossil fuels with hydrogen in current production (11, 12). With hydrogen produced from wind and solar energy, these projects include building large electrolyzers, hydrogen storage, and necessary infrastructure, thereby putting Sweden in the lead of developing green H_2 (13–15).

1.2.2 Ammonia

Ammonia, which is free of carbon, is predicted to be important for decarbonizing shipping and can be mixed with natural gas for electricity and heat generation (Figure 3C) (5). A techno-economic analysis showed that green ammonia produced from biomass via gasification, syngas conditioning, and acid gas removal, is economically feasible. Alternatively, a power-to-ammonia technology using high-temperature solid-oxide electrolyzers is technically feasible but requires decreased stack and electricity costs to become economically feasible (16). Like hydrogen, a larger storage system is required on transport vehicles due to the low volumetric energy density and is unlikely to be deployed in large scale in the near future (Figure 3C) (7). Additionally, combustion of ammonia requires careful regulation of the process to avoid release of NO_x gases, which are potent GHG (7).

1.2.3 Synthetic fuels

Synfuels (methane, methanol, diesel and kerosene) can be produced from hydrogen and CO/CO_2 using Fischer-Tropsch synthesis by gasification of biomass or via electrolysis with fossil-free electricity (5, 17). In the latter method, carbon can be sourced from carbon-capture

technologies and the input energy can balance intermittent electricity supply (17). Also, production of synfuels is estimated to consume slightly less biological resources than production of biodiesel and improve the electrical system flexibility (17). The high energy densities of synthetic hydrocarbons make them attractive for aviation and shipping. However, the technology is still at the research stage (17). Besides, the additional steps required to convert hydrogen and captured CO_2 into synthetic hydrocarbons increase the costs and are likely only feasible in the distant future where carbon prices are high or stricter climate policies are implemented (8).

1.2.4 Biogas and biomethane

Biogas can be used for power generation in combustion engines and combined heat and electricity generation in industrial plants (8). It is produced already today by anaerobic digestion of organic waste (e.g. manure and biogenic municipal waste), by landfills, or in the industrial processing industry (18). Biomethane is generated by upgrading biogas (e.g. by CO_2 scrubbing or methanation) or by biomass gasification-based routes and can be used as a drop-in fuel in gas networks for industry, buildings, and transport (19). The use of biomethane is predicted to increase in the near future (Figure 3B) (5).

1.2.5 Liquid biofuels

Liquid biofuels, such as ethanol and biodiesel, are predicted to play a significant role in reducing CO_2 emissions in the transport sector (Figure 2 and Figure 3A). In 2020, biofuels accounted for 4% of the global transport energy demand (5). In the Net-Zero Emissions by 2050 scenario, the biofuel demand is expected to increase four-fold in the coming decade, especially for use in road transport, and grow slower after 2030, shifting to shipping and aviation. In 2050, liquid biofuels are expected to supply 45% of the global aviation fuel and 14% of the global transport energy (Figure 3). To keep up with this, a large-scale biorefinery needs to be built every tenth week in the coming decade (5). Another study estimated a much higher supply, up to 42%, of the total energy in the transportation sector in 2075 (9). Notwithstanding the need for increasing the production of biofuels, the joint OECD-FAO Argicultural Outlook 2021-2030 predicts a slow growth in biofuels demand in the USA and EU in the coming decade

because of declining governmental support (20). To counteract this trend, the industry needs to provide cost-competitive technologies for biofuel production and become selective on the feedstock choice (e.g. the EU Renewable Energy Directive II is classifying palm oil-based biodiesel as unsustainable) (20). In developing countries, the demand on biofuels is expected to increase rapidly due to growth in the transport sector, consumer demand, and domestic policies for blending with fossil fuels (20). These blending policies also exist in developed countries, with requirements up to 27% of ethanol (e.g. in Brazil, although 10% is common elsewhere) and up to 30% for biodiesel (e.g. in Indonesia) (20). The expected growth is likely to be in second-generation or "advanced" biofuels, which are produced from agricultural and forestry residues, or non-edible energy crops (5, 20). Today, most of the biofuels are still produced with first generation processes, i.e. from edible crops, such as sugarcane, corn, and soybeans (5).

Compared to above mentioned technologies for hydrogen, synfuels, and ammonia, the technologies for first-generation liquid biofuels are among the most mature and cost-effective, thereby offering a fast transition to a fossil-free transport sector (7, 9). The key advantages of using biofuels are the high energy density, high similarities to gasoline and diesel, and the possibility to use existing infrastructure and vehicles with no or minimal modifications (5). Moreover, "negative" CO_2 emissions can be achieved by combining production with low-cost, commercially-ready CCUS, since the CO_2 generated from fermentation is relatively pure (21). However, further technological progress in biofuels produced from second-generation feedstocks (non-edible biomass) is needed since these processes are currently less mature and more costly than the first generation (22–24).

The focus of this thesis will be on liquid biofuels, specifically ethanol. As a combustion fuel, ethanol is already deployed, produced by microbial processes from renewable biomass and can be chemically upgraded to larger fuel molecules or chemicals at low cost (24, 25). Today, there are over 500 ethanol plants that produce <2% of the global demand of liquid transport fuels (24). The increasing number of countries with CO_2 reduction policies requiring blending of biofuels into fossil fuels, together with the need to rapidly transition to a fossil-free transport sector, are

expected to increase the demand on ethanol considerably the coming decade (5, 9).

In addition to environmental benefits, production of ethanol and other biofuels can result in several societal and economical benefits. Examples include job creation, improved energy security, water quality, and soil quality, as well as possible increases in food security in developing countries (26–28). Hence, biofuels contribute to several of the United Nations Sustainable Development Goals (SDG), such as climate action (SDG 13) and affordable and clean energy (SDG 7) (29). The sustainability impacts are discussed in more detail below (in section 1.5).

1.3 Circular bioeconomy

Biofuels and other solutions presented in Net-Zero Emissions by 2050 scenario are part of a bioeconomy, which solves many of the challenges in the current fossil-based economy. The European Commission defines a bioeconomy as the "production of renewable biological resources and the conversion of these resources and waste streams into value added products, such as food, feed, bio-based products and bioenergy" (30). As such, it goes beyond mitigating climate change by substituting petrochemicals and fossil fuels. It also concerns food security, health, providing new chemicals and materials, agricultural and marine practices, bioprocessing and biorefinery concepts, industrial biotechnology, ensuring energy access, clean water, sustainable consumption, and economic growth. Hence, it is central to about half of the United Nations Sustainable Development Goals (30, 31).

The term circular bioeconomy (CBE) was coined in the last decade and stems from the concept of a circular economy where the "value of products, materials and resources is maintained in the economy for as long as possible, and the generation of waste minimized" (32). Making a bioeconomy circular thus entails addressing the whole life cycle of bio-based products, including design, raw material sourcing, production, distribution, usage, collection, end-of-life, and materials and energy recovery (metals, minerals, CO_2) (Figure 4). Durability, reuse, remanufacture, repair, recycling (nutrients/organics), biodegradability, and cascading (sequential use of biomass) are emphasized. The CBE aims to valorize biomass, including its residues and waste streams, in sustainable

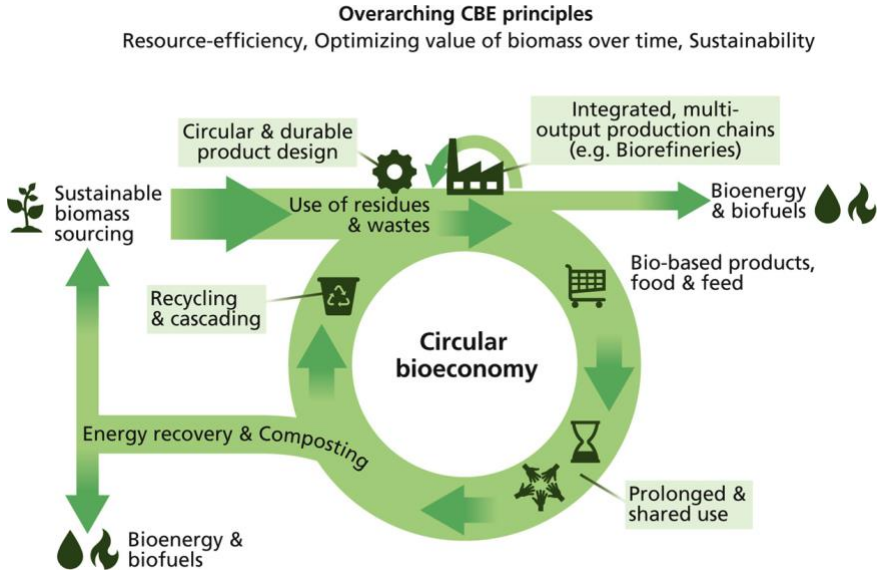


Figure 4. The circular bioeconomy (CBE) and its constituents. Source: Stegmann et al. (34). License: Creative Commons Attribution CC BY 4.0.

and resource-efficient integrated production chains (e.g. biorefineries) to produce bioenergy, biofuels, chemicals, materials, food, and feed (33–35).

1.4 Industrial biotechnology

To realize the promise of the bioeconomy, new process technologies such as those in industrial biotechnology are needed. In general, biotechnology can be defined as "the integration of natural sciences and engineering sciences in order to achieve the application of organisms, cells, parts thereof and molecular analogues for products and services" (36). Industrial biotechnology, also called white biotechnology, is here defined as the use of these biological systems to industrially produce chemicals, materials, and fuels. Furthermore, biotechnology also comprises a medical/health/biopharma sector (red biotechnology), an agricultural sector (genetically modified crops; green biotechnology), a food sector, an environmental sector, and a bioinformatics sector, and these sectors often interface (37, 38). The European Union (EU) identified biotechnology as a *Key Enabling Technology* and priority area for strengthening the EU's industrial and innovation capacity as well as a driver for achieving the

potential of the bioeconomy (39). Similar trends are seen in other regions as well, with USA at the forefront with governmental investments and over 300 large biotechnology companies (38).

Industrial biotechnology offers a sustainable alternative to traditional (petro)chemical processing. By harnessing living cells or enzymes as catalysts, bio-based processes can replace individual or multi-step chemical processes for efficient conversion of carbon sources using fewer solvents, fewer hazardous substances and lower energy intensity. Also, toxic products, by-products, and waste, which are difficult to recycle or degrade, are often avoided (40). Instead of relying on material generated by long geological processes (fossil-based), bio-products are derived from renewable biological material or by direct conversion of CO_2 and H_2. The biological material, here defined as biomass, can come from agriculture, forestry, marine environments, and organic waste streams. In comparison to chemical processes, biotechnological solutions can process the complexity of such feedstocks at a high efficiency and low cost by harnessing the diverse and selective reaction steps contained in the network of evolved biological systems. Similarly, complex products, such as fine chemicals and pharmaceuticals, can be produced in fewer conversion operations with less harsh conditions and high regio- and enantio-selectivity, resulting in cost-effective processes. Compared to conventional chemical processes, these biotechnological processes frequently show increased conversion efficiency, improved product quality, higher reaction rates, and less chemical waste. Such benefits are also seen for bulk chemicals, for instance fuels and plastics. Therefore, switches to bio-technological processes occur in many sectors of the chemical industry and have often been driven by economic advantages, with the ecological benefit as a secondary but important outcome (37, 41, 42).

Concerning CO_2, industrial biotechnology is inherently circular if all parts of the life cycle are based on renewable material and energy. The renewal of biomass follows the crop cycle where CO_2 is assimilated primarily via photosynthesis using the energy from the sun. The produced fuels are combusted into CO_2 that can be assimilated again in the next crop cycle. Similarly, produced chemicals eventually degrade by biological processes or are combusted into CO_2 that is cycled. Thereby, biomass

generation is on the timescale of years in contrast to the million years of geological processes (40, 41, 43).

To be successful, the biotechnological development of industrial bioprocesses requires integration of several methodologies and disciplines, such as microbiology, molecular biology, system's biology, bio-informatics, biochemistry, process technology, and technoeconomic and sustainability analyses. Several successful bioprocesses have already a long history in industry, where the native production of a variety of organic compounds, including fuels, bulk chemicals, and fine chemicals, by microorganisms and enzymes are exploited (44). Discovery of micro-organisms and enzymes in nature that can consume unconventional substrates, produce valuable products, or perform desirable functions, has been aided by omics-based approaches and generated large data libraries. The native abilities of these biocatalysts can be harnessed by utilizing the microorganism or enzyme itself in industrial processes, or by transferring genetic elements into industrial host microorganisms using molecular biology techniques. In either way, the producer biocatalyst usually requires both optimization for high product titer, rate, and yield, and improved robustness for industrial settings, often via protein-, genetic- or metabolic engineering. Moreover, system's biology utilizes omics-based *in silico* approaches built on quantitative physiology to analyze, optimize, and guide strain and bioprocess development. High-throughput screening and testing of engineered phenotypes can be facilitated via synthetic biology tools. Finally, the bioprocess itself can be designed for improved performance, for instance by optimizing the cultivation mode, bioreactor design, scale-up parameters, or product recovery and purification in the downstream bioprocessing (41, 43, 45, 46). Hence, the time-to-market in industrial biotechnology is often long and requires cost-effective sustainable technological solutions that can compete on the market.

1.5 Biorefineries

Biorefineries are industrial biotechnology's equivalent of petroleum refineries, but with renewable biomass as a feedstock instead of oil. As such, the biorefinery is developed and optimized for efficient conversion of biomass into constituent parts and processing these parts into as many

products and value streams as possible, while minimizing waste. Hence, both economic and environmental benefits can be gained.

Biorefineries can be found in different types of industries and span many different feedstocks, products, and process technologies. The feedstocks include dedicated crops on sugar (e.g. sugarcane), starch (e.g. corn), lignocellulose (e.g. wood), oil (e.g. rapeseed), grasses (e.g. grass silage, immature cereals) and marine biomass (e.g. seaweed, algae). Residues can also be used as feedstocks, such as oil-based residues (e.g. animal fat, cooking oil), lignocellulosic residues (e.g. from crops and saw mills) and organic residues (e.g. household waste, manure). The products can be categorized into energy- or material-oriented. Energy products cover fuels (e.g. ethanol, diesel, methanol, synfuels), power, and heat, whereas material products cover chemicals and building blocks (e.g. fine chemicals, amino acids, xylitol, succinic acid, lactic acid), polymers and plastics (e.g. polyhydroxyalkonoates, polylactic acid, cellulose), wood products (e.g. pulp, lumber), fertilizers, food, animal feed, and more. Some chemicals can be used both for energy and as material, e.g. ethanol. Finally, the processes can be categorized based on the type of processing used, as proposed by the IEA Bioenergy Task 42 (47): biochemical (anaerobic/ aerobic cultivation, enzymatic conversion), thermochemical (e.g. gasification, pyrolysis, torrefaction), mechanical/physical (e.g. milling, fractionation), and chemical (e.g. hydrolysis, transesterification, hydrogenation). The biorefinery covers both upstream and downstream processing and can utilize a combination of above methods in separate or integrated factories (47). This thesis will focus on the biochemical processes.

1.5.1 First generation

As mentioned above, different generations of biochemical biorefineries can be distinguished depending on the selected feedstock. First-generation biorefineries utilize conventional feedstocks containing sugar and starch derived from the edible part of food crops. Starch and sugar are commonly originating from sugarcane, corn, cassava, sugar beet, or wheat (20). Today, these biorefineries produce commercially available products, for instance 120 bln L of ethanol globally in 2020, with USA, EU, and Brazil ranking the highest (20).

First-generation biorefineries have raised several concerns on social and environmental sustainability, which has been summarized as the food, energy, and environment dilemma (48). First, it may give economic incentives to clear pristine land and ecosystems (e.g. rainforest, savanna, or grassland) for fuel production, resulting in loss of biodiversity and a carbon debt from the release of sequestered carbon. Second, repurposing fertile agriculture land for fuel production or, in general, expanding fuel production at the expense of food production can have negative effects, such as increased food prices or clearing of pristine land elsewhere to meet the displaced demand. This can indirectly and disproportionally impact poor people, especially in the global South (49–52). For instance, when farmers in the USA shifted from soybean to corn production in 2006 in order to meet the high ethanol demand at the time, a decrease in soybean production lead to increased prices in 2007 (53). Similarly, biofuel production may have contributed up to 30% to the surge in global food prices in 2008 during the global food crisis (53). Other important factors that contributed to this crises were related to demand (financial speculation in agricultural commodity markets, US dollar depreciation, world population growth, expanding middle-class) and supply (increased energy prices, poor harvest, decrease in world grain reverses, restrictions in grain export) (53). The opposite can also occur, where increased sugar or vegetable oil prices due to external market forces can be detrimental to the economic feasibility of first-generation biofuel production, resulting in a shift to producing food instead (51, 54). This has given rise to a public food vs fuel debate, which, in favor of creating an emotive public message that paints victims and culprits, often oversimplifies the issues with land use, food production, and multiple use of crops (49). Several agricultural cases have demonstrated the combined production of food, feed, and bioenergy in integrative, regenerative, and diverse agriculture (49, 55). These more holistic practices and future technological developments could pave a way to biofuels that put this food vs fuel dilemma in the past.

1.5.2 Second generation

As part of such a vision, second-generation biorefineries utilize lignocellulosic feedstocks for production of fuels, chemicals, and materials. These include rotational non-edible energy crops that grow on

marginal lands or poor/degraded soil with high yield (e.g. poplar, willow, eucalyptus, switchgrass, reed canary grass, Napier grass, Bermuda grass, silvergrass), agricultural and forestry residues (e.g. sawdust, wood chips, sugarcane bagasse, rice bran, rice husk, wheat straw, wheat bran, corn stover, forest thinning), and agroindustrial wastes (e.g. potato peel, orange peel, spent coffee grounds, soy bean oil cake, apple pomace, ground nut oil) (56). Because of the natural abundance of lignocellulose, which the plant cell wall is made of, the feedstock cost is low, in some cases even negative (agroindustrial waste), and the utilization of these residues and waste can contribute to a circular bioeconomy (24, 56, 57).

In 2019, more than 40 lignocellulosic biorefineries (excluding food- and wood industry) were operating across Europe producing biofuels, chemicals, materials, electricity, and heat. With peak global investments in cellulosic biofuels around 2011, several facilities were built in the last decade but have had to close due to high costs, low oil prices since 2015, and declining governmental support (24, 58, 59). Less than ten pioneering pre-commercial facilities produced bioethanol in 2019 worldwide (10-30 million gallons per year) (24, 28, 60). With a more beneficial market and demand for biofuels lately, the company Clariant started commercial production of ethanol from agricultural residues in Romania in 2022 (61). This fuel will be sold as a drop-in fuel or for chemical upgrading to sustainable aviation fuels or bio-based chemicals (61).

Several economic and environmental benefits can be gained from using second-generation feedstocks. Model estimations show that replacing first-generation corn with second-generation dedicated energy crops for ethanol production in USA can result in 83% more ethanol per crop area, lower leaching of reducing nitrogen, and 30-470% reduced GHG emissions (i.e. net fixation), due to much smaller need of fertilizer, increased soil organic carbon, and higher harvest volumes (62). A life-cycle analysis on GHG emissions for a “well-to-wheel” case showed that corn-based ethanol reduces GHG with 20-60% compared to gasoline, due to the consumption of natural gas and fossil fuels for the intensive use of fertilizers in current agricultural practices (63). In contrast, second generation ethanol demonstrated essentially net-zero GHG emissions (63). Additionally, second-generation ethanol production can act as a carbon sink, i.e. result in “negative” net GHG emissions, by co-producing

electricity, accounting for soil carbon sequestration, and/or combining with CCUS (21, 63). Another example is growing perennial woody energy crops (such as switchgrass) on former agricultural land. Such a system has several benefits, including immediate GHG reductions, minimal competition with food production, increased biodiversity, improved water quality, and increased carbon sequestration in soils (48, 52). Similarly, growing energy crops between the summer seasons of food crops, i.e. a double crop/mixed cropping system, can minimize food competition and land clearing as well as improve food production by regenerating soil and retaining water and nutrients (48, 55). Finally, sustainable production can also be achieved by using agroindustrial wastes, or agriculture and forestry residues, such as corn stover or wheat straw. Calculations have shown that only a small removal of crop residues from the farmland is sufficient to supply as much bioenergy as dedicated perennial crops (48). Hence, farmers can keep a substantial part of crop residues on the farmland to improve the soil.

Full realization of the environmental and social benefits of second-generation biorefineries will have to take into account more than only optimization of economic profit (64). For instance, acquisition of marginal land may negatively affect poor people that use it for survival. Acquiring higher quality agricultural land or forest can give negative impacts on the environment and food prices. GHG emissions may also vary when removing soil organic content (such as straw), transporting bulky residues, or processing lignocellulose. Using perennials (dedicated energy crops) often improve biodiversity and water quality compared to first-generation feedstocks, whereas second-generation biofuels from food crop residues do not (65, 66). To mitigate this, tools have been developed in order to guide decision making for establishing a second-generation biofuel supply chain while optimizing economic, environmental and social benefits (67–69).

The major challenges of second-generation biorefineries concern supply chain logistics and the processing of lignocellulosic material to monomeric sugars. Due to the bulky and low-density nature of lignocellulosic feedstocks, resource-efficient development of collection, drying, densification, transport, and storage, is needed to realize its economic and ecological value. EU has recently proposed several solutions

including integrated logistical models and establishment of a centralized regional hub (56, 60). Furthermore, the recalcitrant nature of lignocellulose requires use of pretreatment and conversion technologies that result in high capital and operating costs, hence impeding cost-competitiveness (22, 41, 56). These technologies are reviewed in detail in the next chapter.

In this thesis, the focus is on second-generation biorefineries that can make a significant impact and play an important role in reaching net-zero CO_2 emissions and a circular bioeconomy in the near future.

1.5.3 Third generation

Third-generation biorefineries are based on using CO_2 as a feedstock and utilizes microalgae, macroalgae, and autotrophic bacteria. Macroalgae (seaweed) are used today to produce food, feed, and supplements, and have been considered for biofuel production due to their (albeit low) cellulose content (54, 70). Microalgae and photoautotrophic bacteria are naturally rich in lipids and are considered for biofuel production either via transesterification, which yields biodiesel, or via hydrogenolysis producing kerosene-grade alkane that can be used as drop-in aviation fuel (54, 70). They are faster and more efficient than plants, which has led to an exploration *via* metabolic engineering for direct production of fuels and chemicals from CO_2 and solar energy. However, industrial implementation has been impeded by both geographical and technical limitations. Certain regions are less suitable due to the lack of sufficient sun hours, large quantities of water and available sourcing of CO_2 (preferably flue/industrial gas streams). Furthermore, high capital and operating costs are associated with the production process (e.g. sterility in closed systems with engineered strains), the energy-intense lipid extraction and dewatering, and scale up of the cultivation processes (54, 70). These cost drivers might limit future applications of phototropic bacteria to production of bulk chemicals that have low profit margins, such as fuels.

In recent years, a lot of interest has been given to using mixtures of CO_2, CO and H_2 (synthesis gas, or syngas) from flue/industrial/municipal gas streams, which has high CO_2 concentration, lower feedstock costs and uncouples production to land use and light availability (71, 72). These processes produce fuels and chemicals by utilizing chemoautotrophic

microorganisms and could be combined with electrochemical reduction of CO_2/CO to formic acid or methanol (72).

1.5.4 Fourth generation

The use of electricity as an energy source for microorganisms to upgrade CO_2 or other compounds has recently been regarded as an up-and-coming fourth generation of biorefineries (41). This fourth type of biorefinery, that is currently in early development stage, aims to benefit from the future availability of cheap and abundant sustainable electricity as energy source to drive microbial production of fuels and chemicals. With breakthroughs in so called electro-fermentation, an electrode is used as an electron sink or source for microorganisms in order to drive redox reactions that would otherwise be thermodynamically unfavorable (73). The carbon source could be a biomass derivative or captured CO_2. In microbial electrosynthesis, the latter substrate is used together with (renewable) electricity for production of fuels and chemicals (74). These systems are currently still limited by reactor and scaling issues, such as surface-to-volume restrictions, and challenges in finding and engineering suitable microorganisms, metabolic pathways and cathode material for high productivity (41, 73, 75).

2. Cellulosic ethanol production

As described above, the selected combination of product and substrate of this thesis is ethanol produced from second-generation feedstocks, i.e. lignocellulosic material. For a cost-competitive and resource-efficient bioprocess, the process design and attributes of each conversion step need to be considered. The challenge in converting lignocellulosic biomass into ethanol is linked to the recalcitrance and heterogeneous composition of plant material (76). Therefore, biochemical conversion of lignocellulosic biomass into ethanol commonly requires two processes: biomass deconstruction (also called solubilization) into soluble sugars and biological conversion of these sugars to ethanol (77). Both of these processes are characterized by operational and capital costs and require optimization and efficiency in order to maximize profitability and environmental benefits. This section will first address optimization parameters of a general bioprocess, with examples for cellulosic ethanol, and is followed by a description of lignocellulose as a raw material, with the required process steps for solubilization and conversion into ethanol. This chapter ends with the state-of-the-art strategies for optimizing this process.

2.1 Bioprocess optimization

The economic feasibility of a bioprocess will mainly depend on *i*) the capital costs for equipment, *ii*) the operational costs for upstream and downstream processing including energy and materials, *iii*) the cost of raw material, and *iv*) the selling volumes and prices of primary and secondary products. Products can be categorized in low volume/high value, such as fine chemicals and pharmaceuticals, or high volume/low value, including bulk chemicals such as ethanol. The product category will influence the criteria for optimizing a bioprocess and the crucial design parameters. Nonetheless, they often include titer, rate, yield, quality, and robustness, which are described briefly here. It is followed by a description on bioprocess conditions and scale-up that can affect economic profitability.

2.1.1 Titer

The product titer, for instance expressed in grams of product per liter liquid volume in the bioreactor, will influence the downstream processing costs. Lower product concentration will usually require more energy, material and/or larger equipment to concentrate and purify, and thereby increases operational and capital costs. Optimizing the titer is generally very important for low volume/high value products since purification can account up to 90% of the total costs. Even for high volume/low value products, where purification often accounts for a smaller fraction of the total cost, optimizations of the titer can still be crucial for economic competitiveness. Upper limits on titer are often reached due to biophysical (toxicity to the microorganism), thermodynamic, or kinetic limitations (78).

For ethanol, which is the product of choice in this thesis, distillation is a commonly used and cheap method. Nonetheless, even for a relatively cheap method like distillation, the titer still has a very large impact on the energy input and size of the equipment needed. Consequently, the cost of ethanol distillation is greatly increased for concentrations below 40 g L^{-1} (24, 79). For that reason, the alternative lignocellulosic ethanol production strategy discussed in this thesis aims for more than 40 g L^{-1} ethanol as a target for economic feasibility (24, 77).

2.1.2 Rate

The production rate, or productivity, describes the amount of product produced per time unit and can be reported as the total productivity, R (e.g. $g_{product}$ h^{-1}); the volumetric productivity, r (e.g. $g_{product}$ $L_{reactor}^{-1}$ h^{-1}); or the biomass-specific productivity, q_p (e.g. $g_{product}$ $g_{biomass}^{-1}$ h^{-1}). It reflects the needed size of the bioreactors and thus the factory, i.e. the capital costs. Moreover, optimizing the productivity of a current process improves efficacy of the production capacity. For bio-based commodity chemicals, a productivity above 2 g L^{-1} h^{-1} is estimated to be the most economical and environmental (80). For lignocellulosic processing, volumetric rates of solubilization are commonly lower than the rate of fermentation, with common product-ivities in the order of 0.4 g L^{-1} h^{-1} (10 g L^{-1} day^{-1}) (24, 81). Values below this rate are likely to be prohibitive to economic feasibility (24).

2.1.3 Yield

The yield of product on substrate (e.g. in g g^{-1} or mol mol^{-1}) will dictate how much raw material is necessary to reach the target amount of product. Hence, it influences the operational costs of the factory. This is most important for high volume/low value products since the feedstock costs can constitute a large part of the total costs (82). For yeast-based industrial processes, an ethanol yield of >90% of the theoretical maximum on fermentable sugars has been realized and is also a reasonable target for second-generation ethanol processes (83).

2.1.4 Quality

The quality of the product is a crucial factor in process design. For fine chemicals such as pharmaceuticals, reaching high quality can have a higher impact on the process design than the titer, rate, and yield. Although purity is often used as a definition of quality, other parameters such as contamination levels, biological activity, or structural integrity, may be more suitable depending on the product attributes and application. The consumer markets of foodstuff, drinks (such as ethanol), and pharmaceuticals, often have strict requirements on safety. In the chemical industry, a very high purity is required on polymer precursors since impurities can cause discoloration, fragility, and thermal degradation that negatively affect the production process (84). Furthermore, chemical upgrading of biologically-produced building blocks may also require certain purity standards since catalysts can be inhibited and destabilized by biogenic impurities, such as amino acids, proteins, or salts (85–87). In the specific case of ethanol, a very low moisture content is required for fuel combustion, whereas a higher moisture level is acceptable for chemical upgrading (24, 88). Overall, this influences the choice of both upstream and downstream process units and thus affects both capital and operation costs.

2.1.5 Robustness

A robust process can handle variations in process inputs and parameters over time without largely deviating from targeted titer, rate, yield, and/or quality. It will also include risks of process failures, including contaminations, and therefore requires detailed risk assessment and mitigation at each process step. Hence, tools and strategies for designing a robust bioprocess have been developed (89, 90).

2.1.6 Scale-up

Scale-up is a critical step in commercializing a bioprocess that involves possible risks and issues with transferring lab-scale performance to large-scale. Successful scale up of a bioprocess starts with the end in mind, where the final process is conceptualized to great detail at an early development stage. Stepwise increases in scale, from lab (about 0.5-10 L), to pilot (100-10,000 L), and to demo (10,000-100,000 L), promote robust design of large scale, including risk assessment, risk mitigation, technology transfer, and proper evaluation of scale-up parameters (91). In the case of cellulosic ethanol, possible scale-up challenges will likely include mixing heterogeneity (especially in view of the solids content), variations in raw material grade and sterilization outcomes, contamination, culture broth handling (e.g. aerobicity), and changes in hydrostatic pressures that create gradients in partial pressures of gases.

2.1.7 Bioprocess conditions

Two examples of process conditions that impact process economics are gas composition and temperature. Selecting a thermophilic (>45 °C) and non-aerated (anaerobic) bioprocess can provide several economic benefits. In comparison to aerobic growth, anaerobic processes generally have *i*) higher product yields as less of the substrate is converted to CO_2, *ii*) a productivity that is not limited by the oxygen transfer rate, and *iii*) lower operational and capital costs (92). For instance, the minimum selling price of chemicals produced anaerobically decreases with 30% compared to an aerobic process (80). In a case study of cellulosic lipid production, the capital and operational costs increased 5-fold with aeration due to the slurry characteristics of lignocellulose (93). Hence, in view of the low profit margins on cellulosic ethanol, anaerobic processes are more desirable (24).

Furthermore, a thermophilic process will provide cost savings from less intense cooling compared to a mesophilic process. Microbial growth is almost exclusively an exothermic reaction and often requires cooling at industrial scale due to poor heat loss via reactor walls and evaporation. For mesophilic processes (20-45 °C), refrigerated cooling water is often needed, whereas thermophilic processes might allow use of ambient cooling water depending on the geographical climate. The cost savings

from eliminating refrigeration varies. For instance, elimination of a refrigeration system for poly-3-hydroxybutarate (PHB) production from methane by methanotrophs significantly reduced operating costs by a third (94). On the other hand, in cellulosic ethanol production, the cost savings from eliminating cooling was insignificant compared to the other costs of the process (95). However, the perhaps largest advantage with using a thermophilic process in cellulosic ethanol production is that it enables the application of nature's most efficient biomass deconstruction systems, namely those in thermophilic anaerobes, which reduce the costs for pretreatment and enzyme addition as discussed below (24, 77, 96, 97). Additional benefits of thermophilic processes, although not substantiated in economic analyses, are *i*) generally higher reaction rates, *ii*) potentially reduced risks of contamination (which is difficult to verify), *iii*) less intense mixing due to lower viscosity, *iv*) shorter (cooling) time between sterilization and process start, *v*) increased solubility of substrates and products, and *vi*) possibility for *in situ* removal of volatile compounds (98–100).

2.2 Recalcitrance of lignocellulose

Lignocellulose is mainly found in the plant cell wall and consists of 35-50% cellulose, 20-35% hemicellulose, and 5-30% lignin, depending on the plant species and part (77). Cellulose consist of linear chains of β(1→4)-linked D-glucose, which are structured in crystalline cores resistant to chemical and enzymatic hydrolysis (76, 101). Hemicellulose is a branched, amorphous polysaccharide that consist of a heterogeneous mixture of pentoses (C_5 sugars) and hexoses (C_6 sugars) (102). Lignin is a hydrophobic amorphous polymer of non-sugar components, generally aromatics (103). On both a microscopic and macroscopic level, these three components provide the plant cell wall with a structural and chemical barrier, which hampers microorganisms and enzymes from accessing its sugars (76, 77, 103). Although beneficial for plants, the recalcitrance of plant cell wall material is a prominent cost driver in lignocellulose conversion and an important processing challenge to overcome (22, 24, 77).

2.3 Process steps and configurations

The deconstruction of lignocellulose occurs via pretreatment and enzymatic hydrolysis. The aim of the pretreatment is to weaken chemical and physical barriers of lignocellulose by solubilizing hemicellulose and

lignin and increasing the accessible surface area of the cellulose fibers. Thereby, the rate of enzymatic hydrolysis and yield of soluble sugars are improved. The pretreatment can be categorized as either chemical or physical. Chemical methods include treatment with acids, bases, ionic liquids, or solvents, which result in operational costs for addition of chemicals (and/or recycling) as well as a risk of generating degradation products that decrease the yield or inhibits enzymes or microorganisms. Physical methods include *i*) comminutions such as ball milling that mechanically reduces particle size, *ii*) hydrothermolysis, i.e. liquid hot water treatment (e.g. autoclaving) or *iii*) steam explosion. State-of-the-art processes consist of a combination of methods resulting in a harsh thermochemical pretreatment, commonly using dilute acid, steam explosion, and/or chemicals. Pretreatment is followed by the addition of cellulose-active enzymes, called cellulases, often from aerobic fungi such as *Trichoderma reesei* (24, 76, 77, 104).

The process can be divided into five main steps (Figure 5A): *i*) pretreatment, *ii*) enzyme production, *iii*) enzymatic solubilization of cellulose and hemicellulose, which releases hexoses and pentoses, *iv*) separate or combined fermentation of hexoses and pentoses, and *v*) purification by distillation. Depending on if these steps are operated separately or merged, a different number of process units (equivalent to bioreactors) will be needed. In a separate hydrolysis and fermentation (SHF) process, each step has a separate process unit (Figure 5A). Merging solubilization and hexose fermentation in one bioreactor is called simultaneous saccharification and fermentation (SSF), where enzyme production and pentose fermentation are still performed separately. A simultaneous saccharification and cofermentation (SSCF) process has solubilization, hexose and pentose fermentation in one process unit, with separate enzyme production. Besides feedstock costs, separate enzyme production (*in-* or *ex-situ*) and harsh thermochemical pretreatment included in these configurations (and in the state-of-the-art process), constitute the largest costs. Pretreatment is the single most expensive process step, constituting about 20% of the total projected cost (81, 105, 106). The cost of added enzymes is also high with varying estimates of 0.1 (107), 0.3 (106), 0.32 (108), 0.34 (81), 0.35 (109), 0.4 (110), 0.53-0.65 (111), 0.68-1.47 (112) USD per gallon ethanol, which was sold at 1.4-2.7

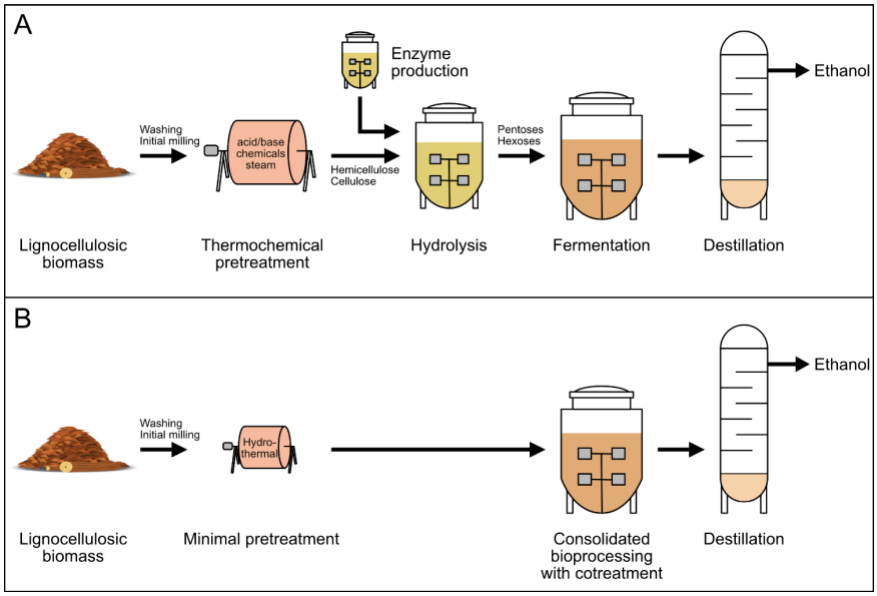


Figure 5. Production process for cellulosic ethanol. (A) The state-of-the-art process with harsh thermochemical pretreatment and addition of fungal enzymes. (B) An optimized process configuration called consolidated bioprocessing with co-treatment (C-CBT). This process utilizes cellulolytic microorganisms that produce their own enzymes and thereby eliminate the costly enzyme production step. Fermentation with the candidate CBP bacterium *Clostridium thermocellum* combined with ball milling has shown >90% total carbohydrate solubilization and reduces the need for costly pretreatment (only hydrothermal) (24).

USD per gallon (nominal prices) between 2010 and 2020 (20, 113). With the state-of-the-art process, the total cost of cellulosic ethanol has been estimated at around 3 USD per gallon (2010 prices) (111). An optimized configuration, called consolidated bioprocessing, uses a mild pretreatment and one process unit for enzyme production, lignocellulose solubilization, and fermentation (Figure 5B) (77). As a result, the total processing costs can be 40% lower than the state-of-the-art process (106).

2.4 Consolidated bioprocessing

CBP utilizes a cellulolytic and fermentative microbial culture, consisting of one or several microorganisms that together produce their own cellulases and ferment sugars to ethanol (77). Compared to added

fungal enzymes, CBP processes with the microorganism *Clostridium thermocellum* demonstrate between two- to ten-fold higher carbohydrate solubilization efficiency on switchgrass, corn stover, corn fiber, and model cellulose substrates using only hydrothermal pretreatment (24). Hence, the pretreatment severity is greatly reduced compared to the state-of-the-art as no catalyst is required and a low-cost reactor design can be used, which together reduce the cost of this otherwise cost-intensive step (114).

Inspired by the alternating bacterial attack and mechanical comminution in the rumination process, CBP can be combined with mechanical pretreatment in so-called co-treatment (C-CBP, see Figure 5B) (96). Ball milling during fermentation with the cellulolytic *C. thermocellum* has shown almost double the solubilization efficiency on switchgrass, poplar, and corn stover, compared to a control without milling (96, 115, 116). Although milling as a stand-alone pretreatment is believed to be impractical due to the high energy intensity, microorganism-mediated solubilization might alleviate this energy demand as the viscosity drops 8-fold in the first 10% of conversion (96, 115–117). A techno-economic analysis comparing *C. thermocellum*-based C-CBP with the state-of-the-art process, showed that C-CBP can offer 43% lower capital cost, 5-fold higher net revenues, and a payback period of 3 years instead of 25 years (22). Interestingly, the cost savings are primarily from eliminating enzyme addition and reducing the severity of pretreatment (22, 106). Therefore, C-CBP becomes an attractive configuration compared to the state-of-the-art technology for cost-competitive cellulosic ethanol production. However, this economic analysis assumed a production of 59 g L^{-1} ethanol at a productivity of 10 g L^{-1} h^{-1} and yield of 88% of maximum, which exceed currently achieved titer, rate, and yield of CBP with *C. thermocellum* (22, 24). Since CBP technology is not as mature as the state-of-the-art process, this analysis is conceptual and requires further research for a proof-of-concept in reaching the industrially required ethanol titer, rate, and yield (24).

Development of CBP microorganisms has followed two strategies: the *recombinant cellulolytic* strategy and the *native cellulolytic* strategy. In the native strategy, cellulolytic microorganisms are engineered for high ethanol production, whereas in the recombinant strategy, non-cellulolytic microorganisms that have favorable ethanol production capabilities are

engineered for heterologously expression of cellulases. For the latter, *Saccharomyces cerevisiae* is the most studied. However, several challenges have been encountered in *i*) reaching high levels of cellulase expression, especially in anaerobic conditions and in the presence of solids, which limit the achievable cell densities, ii) identifying the optimal combination of cellulases that are effective on a range of feedstocks, and iii) demonstrating high solubilization efficiency and rate (118). Challenges in the native strategy consist of less-efficient genetic tools for non-model organisms and achieving industrially-required ethanol titer, rate, and yield (77, 119). Of the two strategies, the native strategy is argued as more promising and likely easier to pursue due to the complexity of replicating the lignocellulose solubilization enzyme machinery of native cellulolytic organisms compared to replicating the ethanol production pathway of ethanologens, as well as the requirement of very high carbohydrate solubilization efficiency for economic feasibility (83).

A promising candidate for the native strategy is the above-mentioned bacterium *Clostridium thermocellum*, for which genetic tools exist and very high lignocellulose solubilization yield and rate have been observed. It outperforms other cellulolytic organisms as well as fungal cellulases in solubilization efficiency, especially in C-CBP with >90% total carbohydrate solubilization (24, 96, 115, 116). However, hitherto, engineering the ethanol pathway in *C. thermocellum* has had limited success. The highest achieved ethanol titer (30 g L^{-1}) (120) and yield on fermentable sugars (75% of theoretical) (121) fall short of the industrial requirements on >40 g L^{-1} at >90% of theoretical maximum yield for economic feasibility (24). Further advancement in the understanding of its metabolism is necessary in order to realize the potential of this CBP-organism (83).

3. *Clostridium thermocellum*

C. thermocellum (*Ruminiclostridium thermocellum*, *Hungateiclostridium thermocellum*, *Acetivibrio thermocellus* (122)) was first isolated in 1926 by Viljoen et al. (123). However, a reliable description was not provided until 1954 by McBee (124) due to difficulties in isolating pure cultures (125–127). The bacterium is a gram-positive, motile, rod-shaped, obligate anaerobe that forms endospores (124, 128). The growth temperature spans between 45 and 68 °C, with an optimum at 60 °C, hence the classification as a thermophile (124, 128, 129). Like other *Clostridia*, the pH span for growth is narrow, between 6.2 and 7.7, and an optimum is found between 6.7 and 7.0 (128, 130).

3.1 A cellulolytic specialist

From early on, *C. thermocellum* was recognized for its native ability to efficiently solubilize lignocellulose, spurring extensive studies due to its potential industrial applications (131, 132). The well-studied, extracellular, large and complexed multienzyme machinery, called the cellulosome, is attached to the cell surface and responsible for the deconstruction of lignocellulose (133, 134). Besides cellulases, these cellulosomes include scaffoldins that secure attachment to the cellulose fiber (77). This efficient organization allows for a synergy between the enzymes in the cellulosome and results in higher activities than that for individual enzymes (77, 133, 135). Additional factors that contribute to the high cellulolytic efficiency are believed to include the unraveling of lignocellulose fiber ends by the cellulosome and synergetic effects from the presence of metabolically active cells, which is still not fully understood (77, 136–139). The attachment results in the formation of a monolayer of cells and the solubilization rate is therefore dependent on the available surface area, in addition to the biocatalyst availability (77, 140, 141). The detailed molecular mechanism of cellulosome-mediated cellulose solubilization is beyond the scope of this thesis (see reviews (77, 134, 135, 138)).

Solubilization and growth have been observed on several types of cellulose-containing substrates. This include model crystalline cellulose (e.g. Avicel) (142), harshly or mildly pretreated lignocellulosic feedstocks such as switchgrass (96, 115, 115, 143), poplar (143–145), corn stover (116), wheat straw (144, 146), sugarcane bagasse (147, 148), cornstalk (149), delignified wood fibers (150), and mushroom compost (151), as well as municipal wastes such as filter paper (148) and paper sludge (152, 153). Upon solubilization, cellulose hydrolysis products are formed, including soluble mono- and oligosaccharides of glucose. *C. thermocellum* has a growth preference for the oligosaccharides, called cellodextrins, above monomeric sugars (154–156). Compared to glucose, growth on oligosaccharides has been shown to be more energetically favorable as the energy stored in the glycosidic bonds is utilized by the cell (155). The preference of *C. thermocellum* for cellodextrins is also shown by long lag times preceding growth on monomeric sugars, such as glucose and fructose (156–160). In industrial settings, this results in undesirable accumulation of hexoses towards the end of the batch and lowers the product yield (121, 142, 161).

Although *C. thermocellum* also solubilizes the hemicellulose fraction efficiently, the microorganism does not grow on, nor use, pentoses (145, 162, 163). The accumulation of monomeric and oligomeric pentoses has been shown to inhibit cellulases as well as growth of *C. thermocellum* (163–166). While engineering *C. thermocellum* for pentose utilization has recently been successful, albeit at low growth rates (167, 168), future CBP processes will likely use co-cultures with pentose-utilizing thermophilic organisms, such as *Thermoanaerobacter* species, *Thermoanaerobacterium* species, and/or other *Clostridium* species, since their synergetic growth offer several benefits. Such co-cultures have shown improved lingocellulose solubilization, reduced residual sugars, and enhanced ethanol titer and yield (146, 169–173). Other benefits for using co-cultures in CBP are cross-feeding of vitamins (174–176), aerotolerant cultures when cultured with facultative anaerobes (177–179), and metabolic shifts toward industrially valuable products such as ethanol, H_2, butanol, or organic acids, when combined with solventogenic, acetogenic, and/or chain-elongating organisms (171, 180, 181).

3.2 A cell factory for production of fuels and chemicals

The heterofermentative nature of *C. thermocellum* results in the production of a myriad of metabolic end-products. The "traditional" fermentation products are ethanol, acetate, formate, H_2, CO_2, lactate, and biomass (182). Recently, also amino acids, especially valine and alanine, have been observed as end-products (182–185). At high initial substrate concentrations of 100 g L^{-1} cellulose, *C. thermocellum* shows "overflow" metabolism that also results in significant production of isobutanol, 2,3-butanediol, and traces of isopropanol, 3-methyl-1-butanol, and 2-methyl-1-butanol (142). This repertoire of metabolic products together with available genetic tools have been harnessed for targeted production of isobutanol (186, 187), n-butanol (187, 188), lactic acid (189–191), H_2 (147–153, 192–194), and recently acetate and isobutyrate esters such as isobutyl acetate (195, 196) and others (197) for which thermophilic conditions ease downstream processing. Although *C. thermocellum* may not be the best candidate for acid production due to its low tolerance to acidic conditions, the organism shows potential for targeted production of amino acids, such as valine, from lignocellulosic feedstocks instead of pure sugars used in today's microbial processes (198). Of these metabolic end-products, ethanol production is the most extensively studied (e.g. (83, 199) and references therein) and closest to commercialization. Metabolic engineering of wild-type has resulted in a titer of 30 g L^{-1} ethanol (120) and 75% of the theoretical maximum yield on fermentable sugars (121), which is close to the >40 g L^{-1} and >90% of theoretical maximum yield needed for industrial implementation.

Underlying the choice in this thesis to focus on ethanol production with *C. thermocellum* as a proof-of-concept CBP, is the idea that technological and knowledge advances that aid this product will in the future likely also aid broader application of *C. thermocellum* as a cell factory for production of other fuels and chemicals, without adding the complexity of (heterologous) product pathways encountered at this early stage. Along this path, fundamental understanding is gathered on the microorganism's physiology, metabolism, and product-related attributes such as tolerance, and help guide metabolic engineering strategies in developing *C. thermocellum* as a second-generation cell factory of a range of products.

3.3 Metabolic engineering

Metabolic engineering was defined by Bailey (1991) as "the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology" (200). It aims at improving production of metabolites with industrial or medical value by optimizing existing metabolic networks and/or introducing heterologous genes or regulatory elements, commonly in bacteria, yeast, or plants (201). As such, metabolic engineering has facilitated development of successfully implemented bioprocesses and constitutes several tools and concepts (78, 200).

About a decade ago, standardized protocols were published for transformation by electroporation and markerless gene editing in *C. thermocellum* (170, 202). This led to several investigations into its fascinating metabolism and improvements of the ethanol titer and yield. With a general understanding of the individual reactions of the fermentation pathways in *C. thermocellum*, the initial strategy was to steer carbon and electron flow to ethanol by knocking out traditional byproduct pathways including formate (203), acetate (170, 183, 204), lactate (170, 183, 205), and H_2 (206), and combinations of them (183, 207). However, this often resulted in growth-impaired strains with at most 70% of theoretical maximum ethanol yield. In contrast, performing these types of knockouts in another thermophilic bacterium with a similar metabolism, called *Thermoanaerobacterium saccharolyticum*, resulted in 88% of theoretical yield (on xylose) (208). This lead to diagnostic studies of the individual steps in the *C. thermocellum* ethanol pathway (187, 189, 209), e.g. by replacing it with the *T. saccharolyticum* pathway (210, 211) and replicating the highly efficient pathway in yeast (212). This has pointed to gaps in knowledge on redox cofactor balancing and complex interactions between the pathways in *C. thermocellum* (83, 213). Concomitant biochemical and functional analyses showed that *C. thermocellum* has an atypical Embden-Meyerhof-Parnas (EMP) glycolysis and fermentation product pathways with high diversity in energy carriers (ATP, GTP, PP_i) and redox cofactors (NADPH, NADH, ferredoxin) (155, 214–217). Although this flexibility is likely to be energetically beneficial for the cell, it has impeded rational engineering (83).

Further improvements in ethanol titer and yield were achieved through laboratory evolution. As a tool, laboratory evolution follows the principles of natural variation, mutation, and selection, and can enrich desirable traits by careful design of the cultivation conditions (218, 219). Elucidating the molecular basis for the increased fitness is often aided by omics-based approaches and reintroduction of identified mutations into wild-type in so-called reverse, or inverse, metabolic engineering (220–222). Such approaches allow transfer of those beneficial elements to other hosts and generally advances knowledge on cellular physiology. Laboratory evolution can complement rational design approaches for improving the phenotype of mutants that show induced cellular stress (219). This tool has had great success in improving microorganism's metabolite tolerance, growth rate, substrate range, and product titer and yield (218, 219). Similar success has been seen with *C. thermocellum* where laboratory evolution of the growth-impaired mutant with knock-outs in the traditional byproduct pathways, resulted in significantly improved growth rate, 75% of theoretical yield, and 22 g L^{-1} ethanol (121). Another study reached an even higher ethanol titer of 30 g L^{-1} with a similarly evolved strain (120). Surprisingly, these strains convert up to 10% of the carbon to amino acids, mainly valine and alanine, which limit the ethanol yield (120, 121). This unexpected and still not understood phenomenon impedes further improvements of the yield (185). In other studies, laboratory evolution has improved the ethanol tolerance of *C. thermocellum*. In comparison to wild-type, which tolerate up to 20 g L^{-1} (129, 187), evolved strains could tolerate >50 g L^{-1} ethanol (223–226). The reason for this so-called titer gap between what *C. thermocellum* can produce (30 g L^{-1}) and tolerate (>50 g L^{-1}) is still not fully understood, but is hypothesized to be more related to thermodynamic limitations than biophysical limitations (83, 119, 227).

In order to better understand the central metabolism and guide engineering efforts, metabolic engineering strategies utilize system's biology and stoichiometric models (228, 229). With an available genome sequence and omics-based techniques, expression data have been collected on the transcriptome and proteome of *C. thermocellum* (121, 230–234). Several models have been built, including kinetic core models that aid understanding on metabolic control and regulation (235, 236), and

genome-scale metabolic models that help assess and optimize the impact of gene deletions and insertions (237–239). However, these models are imprecise due to inaccuracies in genome annotation (gene-protein-reaction relationships), redundancies amongst redox balancing pathways, partial annotation of the genome (240), lack of experimental biochemical characterization of cofactor usage, and incomplete understanding of regulation on *in vivo* activities. Hence, the predictive capabilities of these models would benefit from improved knowledge on the metabolism of *C. thermocellum*.

4. Present investigation

4.1 Aim

The aim of this thesis was to provide new physiological, functional and biochemical knowledge on the central metabolism of *C. thermocellum* to guide metabolic engineering efforts in increasing ethanol titer and yield.

Important limitations on the ethanol yield were addressed by studying substrate utilization (**Paper I**) and byproduct formation (**Paper II**) using metabolic engineering tools. In the former, the genetic changes necessary for reproducible and constitutive growth on hexose sugars, namely glucose and fructose, were investigated with laboratory evolution and reverse metabolic engineering. Byproduct formation of amino acids was investigated by nutrient-limited chemostats in combination with knock-outs of NADPH-supplying and -consuming pathways regulated by ammonium. Furthermore, this thesis addressed hypotheses on potential biophysical and thermodynamic limitations on the ethanol titer. The role of possible pyrophosphate (PP_i)-sources for driving the PP_i-dependent glycolysis of *C. thermocellum* **(Paper III)** and the chaotropic effect of ethanol **(Paper IV)** were investigated. In order to study potential PP_i-sources, knock-out studies combined with functional annotation and physiological characterization were conducted. The chaotropic effect of ethanol was investigated by physiological characterization at varying ethanol concentrations and temperatures.

4.2 Summary of materials and methods

This section provides a summary of the materials and methods used in this work and aims to inform on the design choices as well as the basics of the selected methods.

4.2.1 Strains

Several strains of *C. thermocellum* have been studied over the years with most of the literature focusing on DSM 1313 (previously known as LQ8) (see (127) for a historical description). DSM 1313 has been well

characterized for its physiology, cellulolytic capability, and enzyme biochemistry (83). Around 2010, its genome sequence was made available and resulted in the development of a genetic engineering system, thereby enabling rational metabolic engineering (202, 204, 241, 242). For these reasons, this strain was selected for studying limitations on titer and yield in this thesis. In addition, a genetically tractable DSM 1313 strain, which is called LL345 and developed by Argyros et al. (170), was used.

4.2.2 Strain construction

An important tool in metabolic engineering is rational and targeted DNA recombination that can alter the metabolic or regulatory network to improve production and/or gain better understanding on cellular physiology. The genetic toolbox for *C. thermocellum* includes promoters at different strengths (243) and thermophilic selection markers that allow markerless gene deletion (170, 202). The state-of-the-art gene editing protocol consists of several rounds of selection and counter-selection and relies on the native (low-efficiency) homologous recombination machinery (202).

4.2.3 Media and cultivation

Choices of medium design and cultivation conditions are important for physiological characterization of wild-type and engineered strains. In strain construction, a rich complex medium with yeast extract is often used to support growth by providing a wide range of biosynthetic precursors and thereby reducing the energy requirement for biosynthesis. For *C. thermocellum*, this medium is called CTFUD (202). In quantitative physiology studies, a defined medium is commonly selected in order to separate the effect of the supplied carbon and energy source from the effect of unknown components. Here, the defined low-carbon (LC) medium developed by Holwerda et al. (244) with minimal background carbon was used. This medium was buffered around 7.4 using MOPS and phosphate buffers. In pH-controlled bioreactors, MOPS was excluded and the pH was maintained at 7. Additionally, since this thesis focused on the central metabolism rather than the cellulolytic capabilities of *C. thermocellum*, soluble model substrates were selected instead of lignocellulosic feed-stocks. For this purpose, cellobiose was used since, of the pure compounds, it is the cheapest and most available of the cellodextrins preferred by *C.*

thermocellum (**Paper I-IV**). In addition, growth on glucose and fructose was explored in **Paper I**.

Another design choice is the cultivation temperature. Although the growth optimum is around 60 °C, most of the literature on DSM 1313 uses 55 °C since that facilitates genetic engineering. In order to allow for comparisons, the work in this thesis predominantly used a cultivation temperature of 55 °C (**Paper I-IV**). Only in **Paper IV** were lower temperatures investigated as a method to mitigate ethanol-induced stress.

Three cultivation modes were used: batch (**Paper I**, **III and IV**), continuous (**Paper I and II**) and growth-arrested (**Paper III and IV**). A batch mode is a common operation mode in industrial production of ethanol as that allows growth at the maximum rate. With the ease of operation and the final application in mind, this cultivation mode is also often used in metabolic engineering studies. Batch mode can be carried out in bioreactors of various sizes (small-, pilot-, large-scale) and allow controlled conditions (e.g. pH) and large sample volumes. For faster screening of strains and conditions, open/closed bottles or flasks at low volume are beneficial. Batch growth consists of several phases. First, the cellular metabolism adapts to the new conditions in a lag phase. For *C. thermocellum*, this phase is very long on glucose and fructose and was the subject of **Paper I**, where the aim was to improve growth on hexoses. The lag phase is followed by an exponential phase where the cells grow at a maximum biomass-specific growth rate. As nutrients are depleted or growth conditions are unfavorable (build-up of toxic compounds, acidic/basic conditions), a stationary phase with constant biomass concentration is reached. Finally, in the death phase, the biomass concentration declines. The stationary phase is utilized in so-called growth-arrest experiments to study product formation uncoupled to growth, such as the fermentative capacity in mutant strains (**Paper III**) or at high ethanol concentrations (**Paper IV**).

Continuous cultures can be used for many purposes. In industry, continuous cultures can offer high productivities in production. In research, continuous cultures are used as tools to quantitatively study cell physiology, often at growth rates that are below the maximum specific growth rate by nutrient limitation at steady-state in chemostats. This can lead to increased understanding on bioenergetic and kinetic parameters, including

effects on the biomass and product yields and rates, e.g. from changing the carbon source (**Paper I**) or modifying the redox-cofactor and nitrogen metabolism (**Paper II**). Chemostats can also be used in laboratory evolution experiments where the selection pressure is applied from the start or increasing over time (218, 219). In **Paper I**, this methodology was used to select for growth on hexoses in the presence of cellobiose. For this purpose, bioreactor systems are used to control in- and outflows of gas and liquid, cultivation parameters such as pH and mixing, and allow extensive sampling opportunities.

Chemostats are operated at a fixed dilution rate, which at steady state is equal to the growth rate. The dilution rate must be sufficiently high to allow growth above the maintenance rate, but below the maximum growth rate to avoid wash-out. In the *C. thermocellum* literature, dilution rates of 0.01 to 0.16 h^{-1} have been reported with 0.10 h^{-1} most common for comparison between conditions and engineered strains (120, 155, 185, 245). Hence, 0.10 h^{-1} was the selected dilution rate in **Paper I and II**.

Since metabolic engineering strategies often targets many candidate genes or conditions, screening methods with a higher throughput than bioreactors or bottles become important. In **Paper I**, this was achieved by batch cultivations in multi-well microplates, which are incubated in a plate reader that tracks biomass growth.

4.2.4 Enzyme activity assays

In order to evaluate the biochemical effects of metabolic engineering strategies, gene modifications (e.g. insertions, deletions), and to functionally annotate proteins, *in vitro* enzyme activity assays were used (**Paper I-IV**). Such investigations can increase understanding on the functional role of a protein, cofactor specificity, regulatory mechanisms, potential kinetic limitations and/or gene-protein relationships.

4.2.5 Sequencing

Sequencing of DNA is readily used in (reverse) metabolic engineering and molecular biology for assessment and evaluation. In molecular biology, sequencing is used to confirm correct plasmid assembly, intact constructs and successful genetic engineering. For this purpose, either the targeted locus is locally sequenced by Sanger sequencing (**Paper I-IV**) or next-generation sequencing (e.g. Illumina) can be used to screen the whole

genome for targeted and secondary mutations (**Paper I and III**). In the latter case, whole-genome sequencing (WGS) can help describe the phenotype observed. Identified mutations can be single-nucleotide variations (SNP), insertions and deletions (indels), or duplications of parts of the genome. The SNPs can result in synonymous and non-synonymous mutations. Synonymous nucleotide mutations do not alter the amino acid sequence but may still impact transcription, mRNA transport and translation (e.g. by tRNA availability). Non-synonymous mutations can alter the amino acid sequence of the protein in three ways. First, missense mutations lead to a codon that encodes for a different amino acid, which may result in altered activity, structure, stability, regulation, or cofactor specificity. Second, a nonsense mutation results in a premature stop codon and thereby truncates the protein, often resulting in inactivation of the protein. Finally, a read-through mutation is an exchange of the stop codon for an amino acid codon, resulting in longer proteins that also may lose their functionality. Similarly, indels can result in frameshifts that prolongs or shortens the protein.

4.2.6 Modelling

To close the circle of metabolic engineering, modelling helps describe and analyze the cell biochemistry and place the experimental data into context. They facilitate calculations over networks, from core models to genome scale, and can be used to predict and guide engineering strategies (228, 229). In **Paper III**, stoichiometric modelling was used to investigate if the PP_i produced in biosynthesis is sufficient to drive glycolysis. Moreover, genome-scale modelling was used to identify cycles that can produce PP_i. To account for inaccuracies in genome annotation, the model was expanded to include non-annotated enzyme functions. Also, genome-scale modelling on mutant strains was underlying the discussion on redox balancing in **Paper II**.

4.3 Investigations in support of yield improvements

In this section, limitations on the ethanol yield are approached from two perspectives. One approach addressed substrate utilization by enabling reproducible and constitutive growth on hexoses (**Paper I**). In the other approach, the aim was to minimize byproduct formation by investigating the mechanism behind amino acids as fermentation products (**Paper II**).

4.3.1 Increasing substrate utilization (**Paper I**)

One challenge in optimizing *C. thermocellum* for cellulosic ethanol processes at high yield is the accumulation of glucose towards the end of batch fermentations (121, 142, 161). With low profit margins, a price-driven competitive market and feedstock costs corresponding to a large part of the total price of ethanol (22, 24), full utilization of available sugars is an economic driver for industrial implementation (22, 24, 246). Conversion of glucose occurs only after a long lag time up to 200 h (156, 158–160, 247). This diauxic growth is detrimental to the productivity of the process, resulting in inefficient use of bioreactor capacity. Hence, industrial implementation would benefit from improved and reproducible growth on glucose. Interestingly, similar lag times are observed on another hexose sugar, fructose. Since both glucose and fructose are a magnitude cheaper than cellobiose and have higher solubility, they can facilitate certain experiments, such as labeling studies, long continuous cultivations, oligosaccharide transport studies, or growth at very high substrate concentrations. As such, a strain with reproducible and constitutive growth on glucose and fructose would have industrial and scientific applications. While glucose uptake and metabolism have been mapped (156, 159, 214, 247–249), fructose uptake and metabolism is less clear (160, 248–250). It is also unclear whether adaptation or mutation precedes growth on these hexoses (156, 159, 160, 249).

The aim of this work was to achieve reproducible and improved growth on these hexoses and elucidate the molecular basis for an improved phenotype. To select for growth on either hexose in the presence of cellobiose, carbon-limited chemostats with decreasing concentration of cellobiose and increasing concentration of glucose or fructose in the ingoing medium were used (Figure 6A). In the end, *C. thermocellum* grew on the respective hexoses without added cellobiose. Single colonies were isolated (eight from each hexose; Figure 6B) and characterized by microplate cultivation (Figure 6E and F). The strains isolated from the fructose-grown chemostat (called the fructose isolates), showed no lag time on fructose, glucose, or cellobiose, and had a growth rate on fructose similar to the one on cellobiose (Figure 7A-C), whereas the growth rate on glucose was approximately half (Figure 7B). The strains isolated from glucose-grown chemostats (called the glucose isolates) showed no lag time

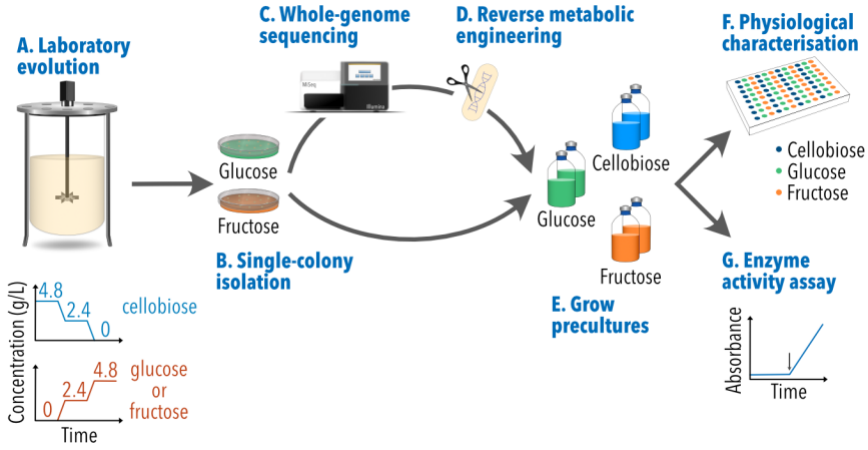


Figure 6. Experimental design of **Paper I**.

on glucose and a similar growth rate on glucose as the fructose strains (Figure 7B). They showed varying lag times and slower growth on cellobiose and fructose (Figure 7A and C). In order to investigate if the isolates had adapted or evolved, they were grown on cellobiose in two serial transfers first (Figure 6E) and then transferred to the hexoses (Figure 6F). Whereas wild-type showed a consistent lag time of 40h on glucose and >80 h on fructose (Figure 7E and F), the isolates showed no lag time (Figure 7E and F). This demonstrated that the improved growth on hexoses was transgenerational and constitutive and suggested that the isolates had acquired mutations that allowed immediate growth on the hexoses.

In order to investigate the underlying molecular mechanisms, whole-genome sequencing of isolated strains and reverse engineering into the wild-type were performed (Figure 6B and C). This revealed two mutations that together reconstituted the phenotype of the fructose and glucose isolates (Figure 8). Inactivation of the gene *clo1313_1831*, encoding for a putative transcriptional regulator and kinase in the Repressor-Open reading frame-Kinase (ROK) protein family (251), abolished the lag time on glucose and resulted in a reduction of the lag time on fructose to about 10 h (Figure 8). In this knock-out, fructokinase activity was derepressed (Figure 8), indicating that phosphorylation might be limiting growth on fructose in wild-type. Surprisingly, ROK did not regulate the glucokinase activity, suggesting another regulatory role likely in the transport of

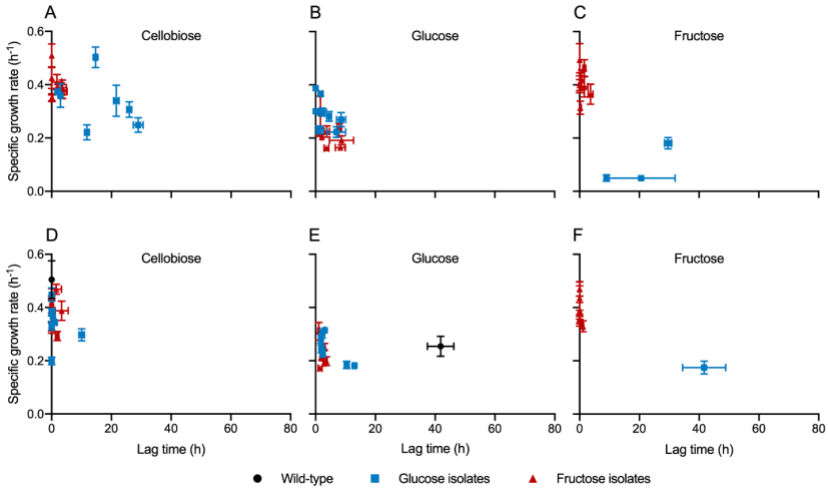


Figure 7. Specific growth rate and lag time of single-colony isolates from glucose-limited (blue squares) and fructose-limited (red triangle) chemostats. Growth on cellobiose (A, D), glucose (B, E), and fructose (C, F) was evaluated for inocula grown on monomeric sugars (glucose for glucose-isolates, fructose for fructose-isolates) (A-C) or grown on cellobiose (D-F) to investigate if the strains were adapting or evolving on the monosaccharides. Wild-type (black circle) was used as a reference and the inoculum was grown on cellobiose. Data are shown as mean ± standard deviation for 3-5 biological replicates of the isolates and 20 biological replicates for wild-type.

glucose. Using transcriptomics to elucidate the targets of this putative ROK transcriptional regulator would be of relevance in future studies. Since the growth rate of the Δ*rok* mutant on fructose (0.11 ± 0.01 h^{-1}) was low compared to the fructose isolates (0.41 ± 0.06 h^{-1}), combinatorial mutations were investigated. Only by inserting a point mutation in *cbpA* (G-to-V substitution in position 148) was the growth rate on fructose reconstituted, with concomitant removal of the lag time on fructose (Figure 8). This gene encodes for a sugar-binding protein belonging to an ABC-transport system (230, 252). The point mutation may have increased the affinity for fructose or altered a signaling cascade for fructose metabolism. In order to investigate the molecular basis of such a mutation, future studies could focus on transport assays combined with transcriptomics and/or proteomics. Overall, these findings together with further expression studies

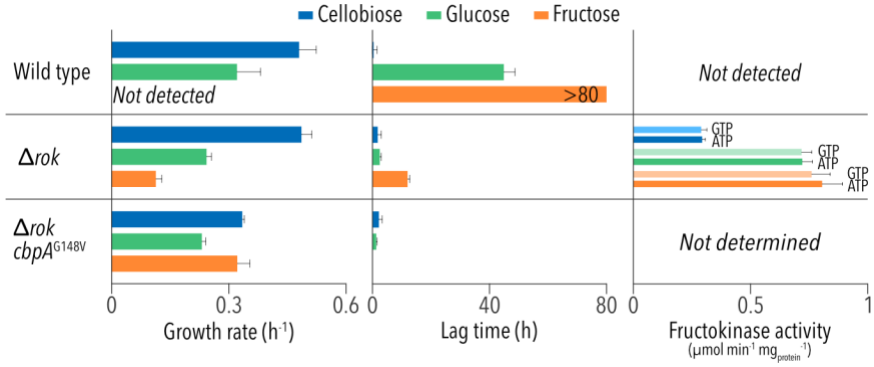


Figure 8. Physiological characterization and fructokinase activity of wild-type, a Δ*rok* strain, and a Δ*rok cbpA*^{G148V} strain. The *rok* gene (*clo1313_1831*) encodes for a putative Repressor-ORF-Kinase (ROK) protein belonging to a family of kinases and transcriptional regulators (251). The cbpA gene (*clo1313_1828*) is a periplasmic sugar-binding protein and is part of an ATP-binding cassette transport system (230, 252).

might help advance the knowledge on the regulation of metabolism on a wider range of substrates by *C. thermocellum*.

In conclusion, *C. thermocellum* was successfully evolved for reproducible and constitutive growth on glucose and fructose. Two underlying mutations were identified with roles in (regulation of) transport or metabolism of these hexoses. These genetic elements can serve as tools both in academia for enabling certain experiments at low cost and in industry for maximizing the feedstock utilization and thereby the ethanol yield.

4.3.2 Minimizing secretion of amino acids (**Paper II**)

Whereas **Paper I** focused on optimizing substrate uptake, **Paper II** focused on understanding amino acid byproduct formation. The significant amounts of extracellular amino acids, which are observed in both wild-type and engineered strains, divert both carbon and electrons away from ethanol formation (120, 121, 142, 183). Engineering for further improvement of the ethanol yield would be aided by better understanding of this unexpected secretion. A hypothesis that the excess of available nitrogen in the batch medium might increase amino acid secretion, was investigated by Holwerda et al. (185) by cultivating *C. thermocellum* in nitrogen-limited

chemostats. Surprisingly, the opposite effect was observed, with several folds increase in specifically valine yield (185). Instead, another hypothesis related to redox-cofactor balancing seems more likely (183, 185). Investigating this hypothesis requires better understanding of sources and sinks of especially NADPH and the regulation of the involved pathways by, amongst others, ammonium. The aim of **Paper II** was to study the role of NADPH-supplying and -consuming pathways regulated by ammonium in order to address potential mechanisms underlying amino acid secretion.

In *C. thermocellum*, NADPH is mainly formed in the malate shunt, which converts PEP to pyruvate via combined action of PEP carboxykinase (PEPCK), NAD-dependent malate dehydrogenase (MDH) and NADPH-dependent malic enzyme (MalE) (214, 215, 253, 254) (Figure 9A). Alternatively, pyruvate is formed via pyruvate phosphate dikinase (Ppdk) with PP_i and AMP as cofactors (214, 254). Interestingly, both MalE and Ppdk are activated by ammonium but with different activation constants (185, 215). Hence, it has been suggested that at lower intracellular ammonium levels, as is likely under nitrogen-limitation, a flux redistribution between Ppdk and MalE may occur that favors NADPH production. If this flux distribution results in an oversupply of NADPH, increased amino acid production, such as valine, could act as a NADPH sink.

To investigate such regulation by intracellular ammonium levels, mutant strains relying fully on either the malate shunt (Δ*ppdk*, called AVM003) or a redox-independent PEP-to-pyruvate conversion relying on heterologously expressed pyruvate kinase (Pyk) (Δ*ppdk* Δ*malE::*P_{eno}*-pyk*, called AVM064) were constructed and investigated along with a reference strain (LL345) in chemostat cultures limited in either ammonium (N-source) or cellobiose (C-source) (**Paper II**). When comparing ammonium limitation with cellobiose limitation, the reference strain showed a 4.5-fold increase in the total amino acid yield, mainly due to increases in pyruvate-derived valine (20-fold), isoleucine (8-fold), and leucine (3-fold) (Figure 10), which is consistent with the study by Holwerda et al. (185). Interestingly, AVM064 (Δ*ppdk* Δ*malE::*P_{eno}*-pyk*), which has a redox-independent PEP-to-pyruvate conversion, only showed a 1.4-fold increase in total amino acid yield and much lower amino acid yields compared to the reference strain (Figure 10). In line with a switch from using the malate

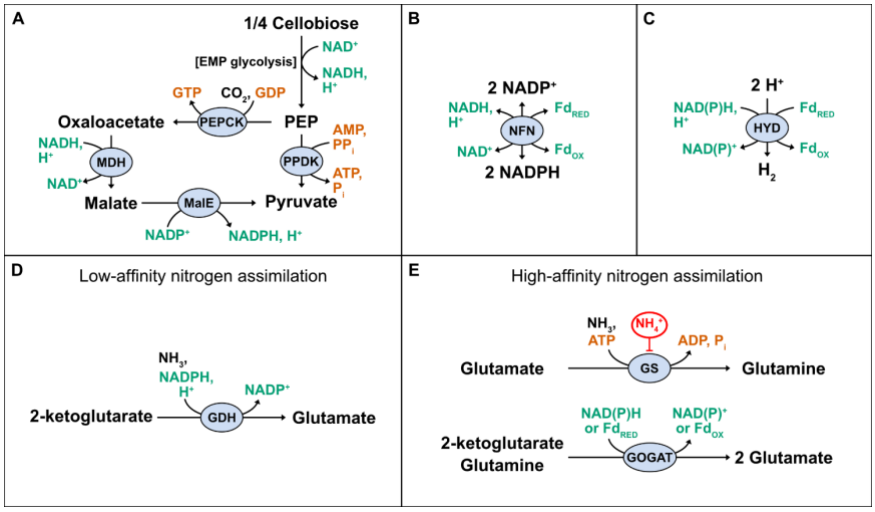


Figure 9. NADPH-dependent pathways in *C. thermocellum*. A: the PEP-to-pyruvate conversion via either Ppdk or the malate shunt, consisting of PEPCK, MDH, and MalE that together transfer electrons from NADH to $NADP^+$. B: the NADH-dependent reduced ferredoxin:$NADP^+$ oxidoreductase reaction. C: a summarized hydrogenase reaction. D: the low-affinity glutamate dehydrogenase reaction. E: the high-affinity ATP-dependent glutamine synthetase-glutamate synthase cycle. Abbreviations: EMP, Embden-Meyerhof-Parnas; PEP, phospho-enolpyruvate; Ppdk, pyruvate phosphate dikinase; PEPCK, PEP carboxykinase; MDH, malate dehydrogenase; MalE, malic enzyme; NFN, NADH-dependent reduced ferredoxin:$NADP^+$ oxidoreductase; HYD, hydrogenase; GDH, glutamate dehydrogenase; GS, glutamine synthetase; GOGAT, glutamate synthase.

shunt, which transfers electrons from NADH to $NADP^+$, to the redox-independent PEP-to-pyruvate conversion in the *pyk* dependent strain, the ethanol yield increased by 54-57% to about 1.6-1.7 mol $mol_{cellobiose}^{-1}$ in either nutrient limitation compared to the reference strain. Hence, these observations suggest that the ethanol yield is limited by NADH availability and that amino acid sec-retion is linked to the NADPH supply. Switching to a redox-independent PEP-to-pyruvate conversion or including a NADPH-dependent ethanol pathway might be effective strategies to improve the ethanol yield.

If the previously observed increased amino acid secretion by wild-type under nitrogen limitation is indeed caused by a redistribution of the

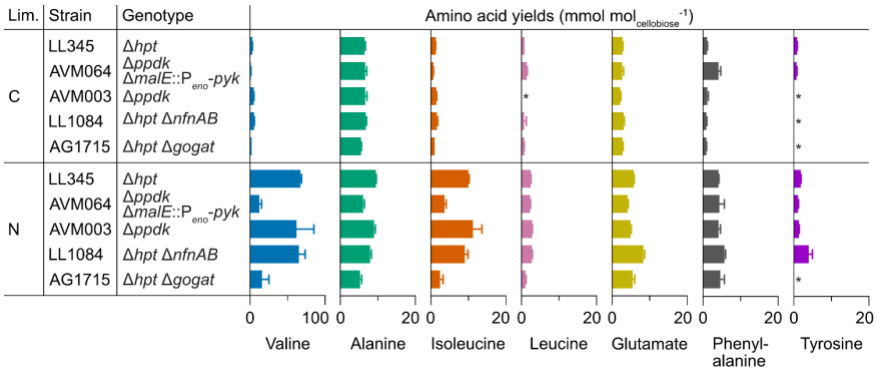


Figure 10. Extracellular amino acid yields in cellobiose (C) or ammonium (N) limited chemostats at a dilution rate of 0.1 h^{-1}. Error bars signify standard deviation for biological triplicates. *<0.5 mmol $mol_{cellobiose}^{-1}$.

fluxes around the PEP-pyruvate node favoring the malate shunt, amino acid secretion should be high and independent of the nutrient limitation in the strain solely relying on the malate shunt. However, the malate shunt-dependent AVM003 (Δ*ppdk*) showed the same amino acid yields as the reference strain in both limitations (Figure 10). This indicates a more complex underlying mechanism than only ammonium-concentration dependent flux redistribution at the PEP-pyruvate node.

Another potential source of NADPH is the NADH-dependent reduced ferredoxin:$NADP^+$ oxidoreductase (NFN, encoded by *nfnAB*) (Figure 9B). NFN catalyzes the reversible transfer of electrons from NADH and reduced ferredoxin to $NADP^+$. In order to exclude possible involvement of NFN in amino acid secretion, a knock-out strain LL1084 (Δ*nfnAB*) was characterized in cellobiose- and ammonium-limited chemostats. The amino acid yields were the same as wild-type in both conditions (Figure 10), thereby suggesting minimal contribution of this enzyme or full functional complementation by other NADPH supplying routes.

In addition to regulating Ppdk and MalE, ammonium also commonly regulates nitrogen-assimilating pathways, such as the lower affinity NADPH-dependent glutamate dehydrogenase (GDH) and the higher affinity glutamine synthetase (GS)-glutamate synthase (GOGAT) (Figure 9D and E). Both GDH and GS activities have been confirmed in *C. thermocellum*, where the GS activity is regulated by the ammonium

concentration (255). Previous attempts to measure GOGAT activity with NADH and NADPH have failed, which is, albeit unconfirmed, in line with the genome annotation of putative ferredoxin-linked activity (255–257). A shift from GDH to a putatively ferredoxin-linked GOGAT would decrease the NADPH reoxidized in the amino acid pathways. For instance, the valine biosynthesis pathway would reoxidize one NADPH instead of two. In other words, regeneration of the same amount of NADPH would require a doubling of the valine secretion flux, possibly contributing to the observed increased amino acid secretion under nitrogen limitation. To this end, a Δ*gogat* strain was constructed (LL1715) and characterized in cellobiose- and ammonium-limited chemostats. With heavily upregulated NADPH-dependent GDH in ammonium limitation compared to the reference strain, lower amino acid yields were expected. This was confirmed in ammonium-limited chemostats with this strain (Figure 10). Surprisingly, deletion of *gogat* resulted in a large increase of the H_2 yield in ammonium limitation, which might reflect an increased need for ferredoxin reoxidation through the hydrogenases (Figure 9C). Together, these observations suggest a ferredoxin-linked GOGAT activity. Concomitantly, ethanol and lactate yields decreased significantly, which is in line with the increased stoichiometric need for NADPH in the ammonium-assimilation and if NADPH formed through the malate shunt goes at the expense of NADH.

Overall, the observations of lower amino acid yields by removing the NADH to NADPH conversion in a malate shunt-deficient strain and by separately changing the co-factor specificity in nitrogen assimilation, show that NADPH has a central role in the observed amino acid secretion. The concomitant changes in ethanol yield in both cases suggest that the NADPH balanced by amino acid biosynthesis goes at the expense of NADH. With lower NADH availability, further conversion of pyruvate to the traditional fermentation products ethanol and acetate might become limited. Hypothetically, NFN could catalyze the electron transfer from NADPH to NAD^+ and ferredoxin, which could drive ethanol and hydrogen formation. However, the lack of a physiological impact in a knock-out of this enzyme suggests021001294 a minimal role under the tested conditions. Instead, the cell seems to solve this issue by producing pyruvate-derived, NADPH-consuming amino acids. These findings

suggest that further improvements in the ethanol yield would benefit from a redox-independent PEP-to-pyruvate conversion and a NADPH-dependent ammonium assimilation.

4.4 Investigations in support of titer improvements

Mechanisms that contribute to titer improvements were explored in this section. Due to the toxicity of elevated ethanol concentrations, growth and ethanol formation may be thermodynamically and biophysically limited. The roles of these mechanisms were investigated in **Paper III and IV**.

4.4.1 Thermodynamic limitations (**Paper III and IV**)

C. thermocellum seems to utilize several mechanisms to save energy and increase its ATP yield (83). One uncommon trait is the use of PP_i instead of ATP in glycolysis, specifically in the phosphofructokinase (PFK) and Ppdk reactions (Figure 11) (214). This results in the following stoichiometry for glycolysis, expressed in glucose equivalents and assuming all flux goes through Ppdk (83).

$$\mathrm{glucose_{eq}} + 3\ \mathrm{PP_i} + 5\ \mathrm{ADP_{eq}} + 2\ \mathrm{NAD^+} \rightarrow 2\ \mathrm{pyruvate} + 5\ \mathrm{ATP_{eq}} + \mathrm{P_i} + 2\ \mathrm{NADH} + 2\ \mathrm{H^+}$$

Hence, depending on the cost of producing one PP_i, glycolysis could yield more than the typical two ATP-equivalents per glucose equivalent. Higher thermodynamic efficiency of ATP synthesis means that less energy is lost as heat and that glycolysis operates closer to equilibrium (258). This is in line with labelling studies showing that the glycolysis of *C. thermocellum* has a smaller thermodynamic driving force than other ethanol-producing bacteria, mostly due to the use of PP_i instead of ATP in the PFK reaction (259, 260). Even low concentrations of ethanol may render glycolysis and ethanol production unfavorable (261).

To understand the impact on the overall ATP yield, the energy cost of producing PP_i needs to be elucidated. PP_i is generated as a waste byproduct in biosynthesis and, in model organisms, hydrolyzed by a cytosolic pyrophosphatase (PPase) to drive biosynthesis strongly forward (262, 263). Organisms that rely on a PP_i-dependent glycolysis, such as *C. thermocellum*, lack this cytosolic PPase and instead recycle the biosynthetic PP_i in glycolysis, which would increase the ATP yield (262,

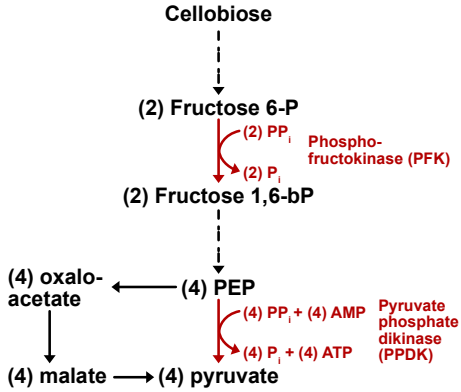


Figure 11. PP_i is used as an energy carrier at two reactions (marked in red) in the atypical glycolysis of *C. thermocellum*.

264, 265). However, stoichiometric calculations in a previous study (214), and updated calculations in **Paper III**, showed that biosynthetic PP_i can only account for up to 5% of the PP_i needed in catabolism (**Paper III**). This was also supported by the observation that a growth-arrested culture could still ferment cellobiose into the traditional end-products (214). While recycling of biosynthetic PP_i may occur, other non-biosynthetic PP_i sources are required to drive glycolysis.

The aim of **Paper III** was to investigate four candidate PP_i-supplying mechanisms in the central metabolism of *C. thermocellum* (Figure 12). First, PP_i can be produced via a reverse membrane-bound pyrophosphatase (mPPase) by using the proton motive force, which is generally generated by a membrane-bound ATPase. Depending on the proton coupling number for the mPPase and ATPase, as well as membrane leakiness, this mechanism could potentially produce one PP_i for less than one ATP, resulting in a higher ATP yield (83). Second, glycogen cycling might generate PP_i in the process of forming and degrading glycogen using ADP-glucose synthase (AGS) (214). This mechanism results in the formation of PP_i and ADP from ATP and P_i. Similarly, a Ppdk-malate shunt cycle can run in reverse to generate one PP_i from one ATP-equivalent. Finally, operation of an acetate cycle could result in the formation of one PP_i from one ATP by forming acetate from acetyl-CoA and degrading it using the key enzyme acetyl-CoA synthetase (ACS). Beyond the recycling in glycolysis of PP_i

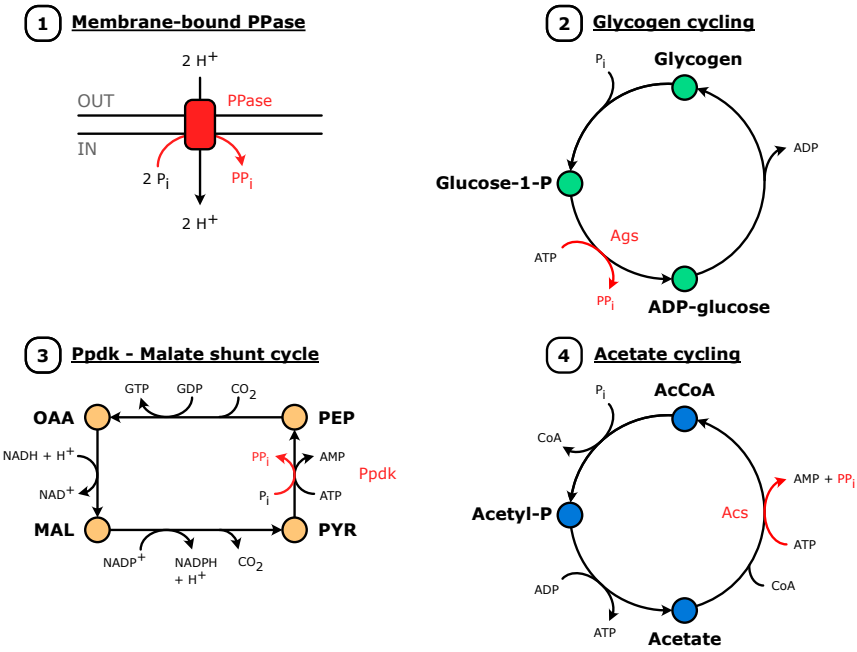
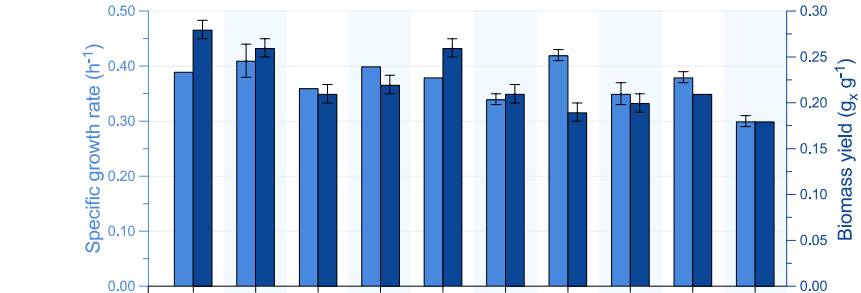


Figure 12. Candidate cycles for supplying PP_i in *C. thermocellum*. Abbreviations: PPase, pyrophosphatase; AGS, ADP-glucose synthase; Ppdk, pyruvate phosphate dikinase; ACS, acetyl-CoA synthetase.

formed in anabolism, only the first mechanism with the mPPase might result in energy savings by using PP_i instead of ATP.

To investigate the contributing role of each PP_i-supplying mechanism, individual knock-outs of the genes putatively encoding mPPase, AGS, Ppdk, and ACS were performed (**Paper III**). The targeted gene modifications were confirmed by *in vitro* enzyme and glycogen assays. Unexpectedly, the annotated *acs* gene did not encode for ACS activity in recombinant *Escherichia coli*, and cell-free extracts of wild-type *C. thermocellum* lacked this activity, making acetate cycling unlikely. It was expected that knocking out a key PP_i-supplying mechanism would be detrimental to the growth rate as PP_i is needed to drive glycolysis. It might also lower the biomass yield if other energetically costlier mechanisms complemented the eliminated activity. Surprisingly, physiological characterization in serum bottles of each individual knock-out did not result in significant changes on the biomass yield or growth rate (Figure



Mechanism	LL1004	AVM008	AVM051	AVM003	AVM059	AVM053	AVM052	AVM060	AVM056	AVM061
1. mPPase		Δ				Δ			Δ	Δ
2. Glycogen cycle			Δ			Δ	Δ	Δ	Δ	Δ
3. Ppdk cycle				Δ			Δ		Δ	Δ
4. Acetate cycle					Δ			Δ		Δ

Figure 13. Physiological characterization of strains with individual and combined knock-outs in key enzymes of four possible PP_i-supplying mechanisms with the wild-type strain LL1004 as reference. Maximum specific growth rate (light bar) and biomass yield (dark bar) are reported as averages of biological triplicates with error bars representing standard deviation. Lack of error bars signify zero in standard deviation.

13). To test a hypothesis on complementarity between mechanisms, combinatorial knock-outs were performed, resulting in double, triple, and quadruple knock-outs (Figure 13). This resulted in a much smaller decrease in growth rate and biomass yield than expected of a mechanism supplying PP_i to drive glycolysis. Parts of this decrease can be explained by reduced metabolic flexibility and the removal of glycogen, which is inexpensive to form compared to other cell components. The possibility for secondary mutations to lower the PP_i demand or alter the intended deletions was excluded based on whole-genome sequencing of mutant strains.

With the conclusion that none of the targeted sources play essential PP_i-supplying role(s), either currently unknown PP_i supplier(s) are operational, or alternative phosphorylating mechanisms exist. The former was addressed by genome-scale model-guided discovery of PP_i-supplying mechanisms (**Paper III**). This resulted in several potential cycles that overlap with essential biosynthetic networks, such as nucleotide formation, or use unavailable precursors. Their contribution was not studied here, partly due to the difficulty in interpreting the physiological effect of

knocking out these essential pathways (if possible), and partly because of the time-consuming nature of current genetic tools to identify the gene candidate encoding these enzymes. Hence, better gene editing tools or another scientific approach are needed for further investigation. The other possibility of alternative phosphorylating mechanisms would mean a lower demand on PP_i in glycolysis. For instance, *C. thermocellum* encodes an ATP-dependent PFK that could reduce the requirement for PP_i. However, with several failed attempts to measure this activity *in vitro* (both in **Paper III** and (214)), its physiological role remains elusive. Overall, further investigation is needed in order to understand the role of PP_i in driving glycolysis and potentially limiting the overall driving force. Such insights could guide metabolic engineering efforts in for instance constructing an ATP-dependent glycolysis.

Several indications suggest that the overall driving force limits the ethanol titer. In laboratory evolution experiments at high ethanol concentration, which has improved ethanol tolerance from 20 g L^{-1} to above 50 g L^{-1} (129, 187, 223–226), the ethanol pathway was mutated, specifically in the bifunctional acetaldehyde/alcohol dehydrogenase gene (*adhE*) (187, 224, 266). Point-mutations in *adhE* were responsible for most of the ethanol-tolerance trait as reintroductions into wild-type resulted in tolerance to 40 g L^{-1} (187, 266). Interestingly, these point-mutations either shifted the cofactor specificity from NADH to NADPH (266) or expanded it to include both NADH and NADPH (187). Since NADPH/$NADP^+$ ratios are higher than NADH/NAD^+ ratios in *C. thermocellum* (267–269), the thermodynamic driving force of this reaction might increase.

Supporting evidence for such thermodynamic limitations was provided in **Paper IV**, where an *adhE* knock-out was used to investigate the contribution of reverse flux on ethanol tolerance. The non-ethanol producing Δ*adhE* strain, called AVM062, overexpressed the native lactate dehydrogenase gene (*ldh*) to balance the NADH previously reoxidized in the ethanol pathway. The ethanol tolerance of this strain improved significantly with higher growth rates compared to wild-type in the presence of >10 g L^{-1} added ethanol and showed an ability to initiate growth at 40 g L^{-1} (**Paper IV**). This adds to the pool of evidence that thermodynamic limitations limit tolerance to ethanol. Important to note is that although mutating *adhE* enhances ethanol tolerance, only minor

improvements in achieved higher titer are observed (187, 209, 224). The role of this mutation in strains engineered for minimal byproduct formation is still unclear (121, 206).

4.4.2 The chaotropic effect of ethanol (**Paper IV**)

As the thermodynamic limitations are unraveled, the biophysical limitations from ethanol toxicity are expected to become more important (83). The biophysical toxicity of ethanol is primarily due to its chaotropic nature, which increases membrane fluidity, denatures nucleic acids and proteins, and effectively disrupts essential biological systems, including membrane-dependent ATP generation and nutrient transport (270, 271). One way to counteract these chaotropic activities is to lower the cultivation temperature, which would rigidify the membrane (270). Such interventions have improved ethanol tolerance in yeast and bacteria and can be used to optimize the bioprocess for higher ethanol titer (129, 272–275).

The aim of **Paper IV** was to investigate if lowering the cultivation temperature could improve ethanol tolerance in *C. thermocellum* and thereby advance the knowledge on the chaotropic effect of ethanol on growth. In order to separate the thermodynamic limitations from biophysical, a non-ethanol producing strain AVM062 ($P_{clo1313\_2638}$*::ldh** Δ*adhE*) was constructed (described above).

In order to evaluate the effect of lowering the temperature, the growth rates of wild-type and AVM062 were compared at 45, 50, and 55 °C in zero to 50 g L^{-1} of added ethanol in serum bottles (Figure 14). A relative growth rate at each temperature was calculated as the ratio between the growth rate in the presence and absence of ethanol. Interestingly, lowering the temperature in the presence of ethanol resulted in higher relative growth rates for wild-type. This indicates that the chaotropic effect of ethanol on wild-type at industrially required titers can be counteracted to some extent by bioprocess optimizations. Surprisingly, this positive effect was not observed for the non-ethanol producing AVM062 (Figure 14), suggesting that the beneficial effect in wild-type is related to the ethanol production pathway. In *C. thermocellum*, this pathway relies on the complex network of redox reactions, including transhydrogenases and hydrogenases, ultimately shuffling electrons between ferredoxin, NAD(H), and NADP(H) involved in the catabolism of cellobiose (83).

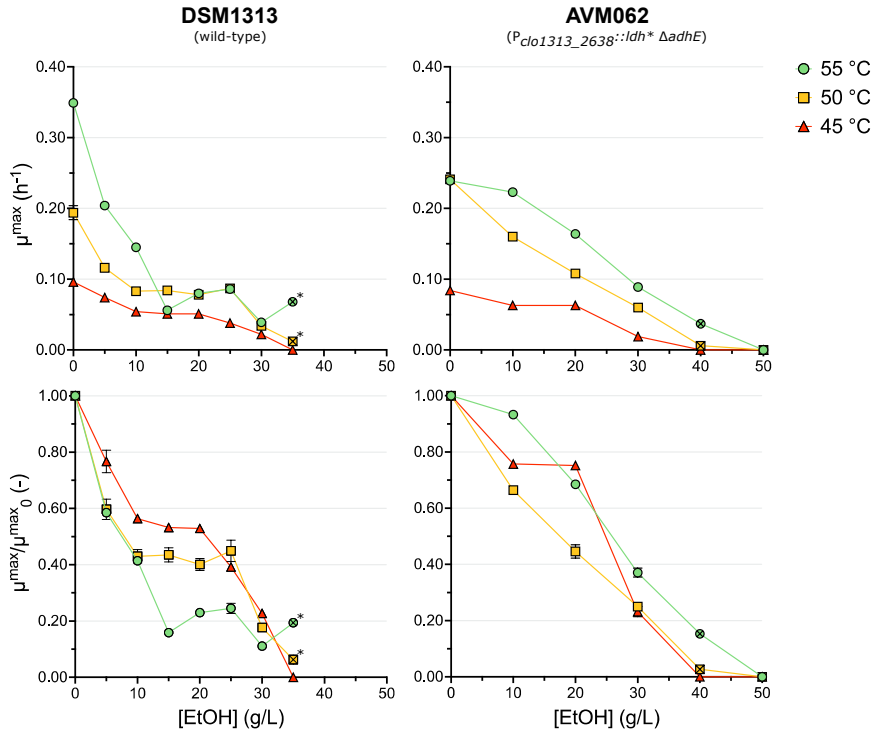


Figure 14. Maximum specific growth rate and relative growth rate of wild-type and the non-ethanol producing AVM062 at different ethanol concentrations and growth temperatures (diamond, 45°C; square, 50°C, circle, 55 °C). The relative growth rate was calculated as the ratio between the growth rates in the presence (μ^{max}) and absence (μ^{max}_0) of added ethanol at the same temperature. The crossed-out symbols indicate that growth was preceded by a long lag phase (> 40 h). Data represent average and mean deviation for two independent biological duplicates.

Since some of these are membrane-bound, e.g. the ion-pumping ferredoxin:NAD^+ oxidoreductase (RNF) and the ferredoxin-dependent energy-converting hydrogenase (ECH) (206, 216), the increased membrane fluidity at higher ethanol concentrations may disrupt their function and thereby impair growth. Besides these chaotropic effects related to the ethanol pathway, the significant improvement in growth rate in the presence of ethanol by simply knocking out *adhE* and over-expressing *ldh*, suggests that thermodynamic limitations are a major mechanism for ethanol inhibition (Figure 14). Due to the low driving force

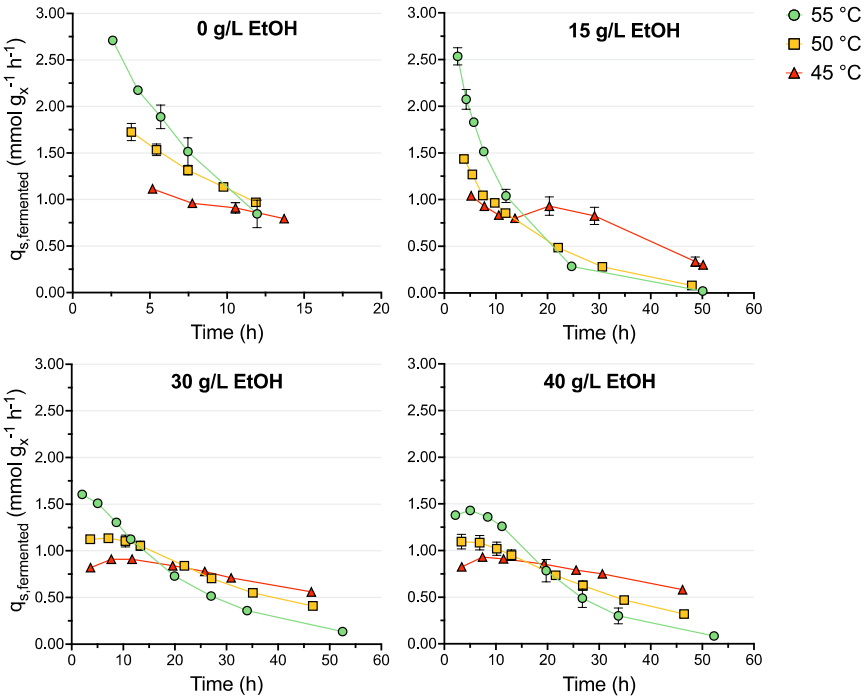


Figure 15. The specific fermentation rate of growth-arrested wild-type plotted against time at different concentrations of added ethanol. It was calculated as the biomass-specific cellobiose consumption rate subtracted by the cellobiose hydrolysis rate (into unconsumed glucose). Data represent average and mean deviation of biological duplicates from the time-point of growth arrest until cellobiose became limiting (>0.5 mM).

in the ethanol pathway, elevated ethanol concentrations likely result in higher NADH/NAD^+ levels, which likely influence the other redox cofactors and could inhibit the involved redox enzymes.

A reoccurring phenomenon observed here and in other studies with high initial substrate concentrations is growth cessation before all the substrate is consumed, followed by continued fermentation (120, 142, 261). Similar to improvements on the membranes, lowering the temperature can also have positive effects on the intracellular proteins of the product-formation pathways, potentially improving the fermentative capacity after growth has ceased (270). This was investigated in **Paper IV** by lowering the temperature for growth-arrested cultures in the presence

of added ethanol. Here, lower temperatures were beneficial for slowing down the decrease of the specific fermentation rate (cellobiose consumption rate corrected for hydrolysis to unconsumed glucose) over time (Figure 15). At 55 °C, the rate was initially higher than at 45 °C but dropped rapidly to levels lower than observed for 45 and 50 °C. In contrast, at 45 °C the rate was more stable and relatively unchanged over time. This suggests that the chaotropic effect of ethanol may impact sugar transport, which is not uncommon (270, 271). The improved fermentative capacity indicates that industrial bioprocesses might benefit from lowering the cultivation temperature at the end of the cultivation after growth has ceased. Such intervention would however also affect the absolute rates and require a cost-benefit analysis where the effect on the final product concentration, total productivity (R, mol h^{-1}), and the operational and capital costs are considered together.

Overall, **Paper IV** showed that both chaotropic effects and thermodynamic factors contribute to the poor ethanol tolerance of wild-type. Moreover, both growth and fermentative capacity of wild-type at high ethanol concentrations are improved by lowering the cultivation temperature. Further studies into the effects of lowering the cultivation temperature with high ethanol producers and on lignocellulosic feedstocks at industrial conditions would aid process optimizations for improved ethanol production. Since the *adhE* knock-out eliminates the role of end-product inhibition and allows studying other factors that influence growth and fermentation, this mutant serves as a good platform strain for further investigations of ethanol toxicity at high lignocellulose loadings, where growth cessation is often observed before the substrate is consumed. Finally, these findings indicate benefits with constructing a simplified (redox) metabolism that does not rely on membrane-bound (trans)-hydrogenases and that has a high driving force, e.g. by engineering an ethanol pathway dependent on pyruvate decarboxylase.

5. Conclusions and future perspectives

This thesis contributes with fundamental insights into the central metabolism of *C. thermocellum* and, additionally, can be applied in metabolic engineering strategies to maximize the ethanol titer and yield of this cell factory for sustainable second-generation bioethanol production.

Paper I demonstrated that *C. thermocellum* can be engineered for reproducible and constitutive growth on glucose and fructose by introducing two novel mutations. The combination of laboratory evolution, whole-genome sequencing and reverse engineering was shown to be an effective tool in uncovering these underlying mutations and improving substrate utilization. The regulation of a hitherto unknown fructokinase, and possibly of glucose/fructose transport proteins, showed that the transcriptional regulator and kinase protein (ROK) plays a central role in repressing growth on hexoses. To elucidate the exact mechanism of ROK regulation, future studies could investigate the transcriptional response upon growth on glucose and fructose in strains with and without active ROK enzyme. With abolished lag time on glucose, the ROK inactivation is a relevant candidate for reducing glucose accumulation in wild-type or engineered strains at high cellulose loadings and thereby improving the ethanol yield. In addition to ROK, a G148V mutation in *cbpA* was important for fast growth on fructose without a lag time. With few protein structures available for this type of membrane-bound carbohydrate-binding protein, *cbpA* of *C. thermocellum* and the mutations found in this study would be interesting for combined study of the protein structure, affinity, and transport, to elucidate genotype-protein-phenotype relationships. This could be complemented with transcriptomic and/or proteomic analysis to gain insights into possible regulations of *cbpA* on fructose uptake. Finally, the strains engineered for growth on hexoses enable knock-out studies and functional analysis of cellodextrin transport proteins (*cbpABCD*) and cellobiose/cellodextrin phosphorylases, investigations into the metabolism at very high substrate concentrations (>120 g L^{-1}) and

reduce costs for stable-isotope-labelling and continuous cultivation studies.

Both **Paper II and IV** pointed to thermodynamic, biophysical and metabolic limitations on the native ethanol production pathway. **Paper II** demonstrated that the availability of NADH likely limits stoichiometric conversion of pyruvate to ethanol. The build-up of pyruvate and NADPH from the malate shunt seems likely to drive production of pyruvate-derived amino acids. Furthermore, the findings of **Paper IV** suggest that the chaotropic effects of ethanol have a large impact on the native ethanol pathway, likely because of its dependency on membrane-bound RNF and hydrogenases. **Paper IV** also showed that the reversibility of the ethanol pathway is a dominant factor in limiting titer and tolerance. Overall, these findings highlight the benefits of a simplified glycolysis and designing an ethanol pathway with a high thermodynamic driving force, independent of membrane-bound proteins and relying on fewer redox carriers. These characteristics can be found in the ethanol pathway in *S. cerevisiae*, which has a redox-independent PEP-to-pyruvate conversion based on PYK and a pyruvate decarboxylase (PDC)-catalyzed NADH-dependent ethanol pathway (276). However, expressing a highly functional PDC in thermophiles has proven to be challenging due to poor thermostability and possible toxicity of the PDC product acetaldehyde at elevated temperatures (212, 277, 278), and is a topic that requires further research.

With strong indications in **Paper II and IV**, and other studies, that the thermodynamic driving force in glycolysis is important for ethanol production, **Paper III** aimed at providing better understanding on mechanisms impacting this driving force and thereby guiding metabolic engineering for higher ethanol titer. By investigating the source, and consequently the energy cost, of PP_i to drive glycolysis, **Paper III** could exclude two previously proposed PP_i-supplying mechanisms (mPPase and glycogen cycling) and two putative model-guided mechanisms (Ppdk-malate shunt cycling and acetate cycling). Hence, it was proposed that currently unknown alternative mechanisms for PP_i supply or alternative phosphorylation in glycolysis must exist, with indications that such mechanisms are unlikely to provide more energy from using PP_i. Future studies could use genome-scale models with the updated biosynthetic PP_i stoichiometry and the physiological data on mutant strains supplied in this

paper, to identify novel mechanisms for PP_i supply. Furthermore, biochemical studies on the annotated ATP-dependent PFK by purifying the protein and test metabolite activators and repressors, would be relevant to shed light on possible alternative phosphorylation in glycolysis. Continued investigation into the PP_i-dependent glycolysis would be beneficial to better understand the evolutionary advantage of using a PP_i-dependent glycolysis, including trade-offs between the thermodynamic driving force and the ATP yield. With these insights, a glycolysis with higher driving force might be engineered to improve ethanol production and tolerance.

In addition to metabolic engineering of *C. thermocellum*, process optimization such as lowering the cultivation temperature can also serve as an intervention to improve ethanol tolerance, which was illustrated in **Paper IV**. Both growth and the fermentative capacity improved by lowering the cultivation temperature at elevated ethanol concentrations. In follow-up studies, batch cultures at industrial conditions with high cellulose loading could be used to test different temperatures on growth, ethanol formation, and fermentation after growth cessation, and be used to design a temperature profile that optimizes the total ethanol production. This could, for instance, consist of a two-stage cultivation where the cultivation temperature is lowered towards the end of the cultivation to push for higher ethanol titer, similar to how yeast-based ethanol production is performed. As such, this intervention requires no underlying knowledge on the pathways or metabolism of *C. thermocellum* and is simple to implement in a bioprocess.

With insights provided in this thesis and other studies, more work is needed to reach the industrial targets on ethanol yield and titer. A hurdle that underlies a major part of this thesis is the laborious and time-consuming gene editing protocol for *C. thermocellum*. Current methodology relies on poor homologous recombination and low transformation efficiency, which slow down hypothesis testing and limits library construction. New tools such as CRISPR-based systems and heterologous recombinases would be beneficial for improved genetic engineering and are currently being developed (279–281). Enabling multiplexing, increasing the efficacy, increasing the throughput, and reducing the time-scale of strain construction, would speed-up research and development of *C. thermocellum*. In addition, expanding the genetic toolbox with inducible

promoters that allow tight and consistent control of gene expression would be beneficial. Such a system could be used to evaluate the physiological role of genes that have not been possible to delete, such as the PP_i-dependent PFK, or to express heterologous proteins that may form toxic intermediates or have poor stability.

Future studies on interventions other than those presented here are also important for realizing consolidated bioprocessing. For instance, a significant body of literature focuses on optimizing co-cultures of specialists, such as homoethanologens, pentose-utilizers, and cellulolytic strains, as this might circumvent some of the challenges of pure cultures with *C. thermocellum*. This type of co-culture is envisioned to solve the challenge of unutilized pentose without requiring extensive pathway engineering in *C. thermocellum*. For this purpose, it will be important to minimize byproduct formation of *C. thermocellum* and ensure that both growth and the cellulolytic ability of *C. thermocellum* can tolerate high ethanol concentrations.

Finally, the findings in this thesis might also be used to develop *C. thermocellum* for production of chemicals and other fuels. Depending on the efficacy of consolidated bioprocessing of lignocellulosic feedstocks, ethanol could contribute to sectors other than transport, e.g. as a material or by chemical upgrading to higher value products. If proof-of-concept cellulosic ethanol production can be delivered, this will likely inspire further development of *C. thermocellum* as a second-generation cell factory for a range of fuels and chemicals and thereby play an important role in a circular bioeconomy and in achieving net-zero emissions.

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