

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

## **Towards Syngas Electro-fermentation**

Utilizing and improving *Clostridium ljungdahlii* as a host for microbial electrosynthesis

CHAEHO IM

Department of Life Sciences  
CHALMERS UNIVERSITY OF TECHNOLOGY

Gothenburg, Sweden 2025

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*“A scientist is happy, not in resting on his attainments,  
but in the steady acquisition of fresh knowledge.”*

- Max Planck



## **Preface**

This dissertation partially fulfils the requirements for obtaining the degree of Doctor of Philosophy at the Department of Life Sciences, Chalmers University of Technology. The work was supported by Swedish Energy Agency grant no. 46605-1 and STINT project no. MG2020-8844 awarded to Associate Professor Yvonne Nygård. The PhD studies were carried out between August 2019 and February 2025 under the supervision of Associate Professor Yvonne Nygård and Professor Carl Johan Franzén, along with co-supervision by Professor Oskar Modin from the Division of Water Environment Technology and Professor Kaspar Valgepea from the University of Tartu, and examination by Professor Lisbeth Olsson.

Most of the work in this thesis was carried out at the Division of Industrial Biotechnology at the Department of Life Sciences at Chalmers University of Technology. Experiments using CO of Paper I were performed in the laboratory of Professor Jung Rae Kim at Pusan National University, Republic of Korea, supported by the STINT project.

Chaeho Im,  
February 2025



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Chaeho Im

Division of Industrial Biotechnology – Department of Life Sciences, Chalmers University of Technology

## Abstract

Gas fermentation technology using acetogenic bacteria couples the capture of greenhouse gases (e.g. CO<sub>2</sub> and CO) with production of value-added chemicals from syngas. However, CO<sub>2</sub> and CO fixation by acetogens *via* the Wood-Ljungdahl pathway requires more reducing equivalents than those present in syngas. The intracellular redox balance of electro-active bacteria can be regulated by a bioelectrochemical system (BES), which combines microorganisms such as acetogenic bacteria with an electrochemical system. The electro-fermentation of CO<sub>2</sub> and CO in a BES has been successfully demonstrated, yet its applicability remains unexplored.

Optimizing the operational parameters for the host strain in a BES is essential to ensure the desired performance. At present, low ATP yield from metabolism, pH sensitivity, and inefficient electron uptake in a BES limits cell growth and product formation of acetogens during electro-fermentation. This can be ameliorated only by detailed understanding of their physiology and extracellular electron transfer.

The aims of this thesis were 1) to identify the most important gas-fermenting bacteria for CO electro-fermentation, and 2) to improve their performance *via* parameter optimization and strain engineering using adaptive laboratory evolution (ALE) and rational design.

*Clostridium ljungdahlii*, which is a proven electro-active acetogen, was selected for microbial electrosynthesis. *Clostridium autoethanogenum*, which is genetically similar to *C. ljungdahlii*, was identified as the dominant species in a CO-enriched culture originating from cow fecal waste and meant for CO electro-fermentation. Low current input (10 mA) in a BES promoted ethanol production and improved cell viability. Operational parameters and culture conditions were systematically tested for improved performance and reproducibility. ALE of *C. ljungdahlii* on iron was attempted to identify and improve potential mechanisms of extracellular electron uptake. Genome sequencing following ALE revealed mutations in membrane-related proteins. A mutant was found to produce ethanol, hypothetically as an energy storage chemical, rather than enhancing extracellular electron uptake and producing acetate. Heterologous expression of pyruvate formate lyase from *Acetobacterium woodii* improved growth and acetate production of *C. ljungdahlii* under H<sub>2</sub>:CO<sub>2</sub> conditions both in the absence and presence of formate as a co-substrate.

The results of this thesis will help ameliorate CO<sub>2</sub> and CO capture by combined gas fermentation and electro-fermentation.

**Keywords:** *Clostridium ljungdahlii*, Gas fermentation, Electro-fermentation, Microbial electrosynthesis, Adaptive laboratory evolution, Pyruvate formate lyase, Metabolic engineering





## List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I. **Chaeho Im**, Minsoo Kim, Jung Rae Kim, Kaspar Valgepea, Oskar Modin, Yvonne Nygård, Carl Johan Franzén (2024). “Low electric current in a bioelectrochemical system facilitates ethanol production from CO using CO-enriched mixed culture”, *Front. Microbiol.* 15:1438758. DOI: [10.3389/fmicb.2024.1438758](https://doi.org/10.3389/fmicb.2024.1438758)
- II. **Chaeho Im**, Kaspar Valgepea, Oskar Modin, Yvonne Nygård (2022). “*Clostridium ljungdahlii* as a biocatalyst in microbial electrosynthesis – Effect of culture conditions on product formation”, *Bioresour. Technol. Rep.* 19:101156. DOI: [10.1016/j.biteb.2022.101156](https://doi.org/10.1016/j.biteb.2022.101156)
- III. **Chaeho Im**, Kaspar Valgepea, Oskar Modin, Yvonne Nygård, Carl Johan Franzén (2025). “Different growth pattern during microbial electrosynthesis using *C. ljungdahlii* evolutionary adapted on iron”. (*manuscript*)
- IV. **Chaeho Im**, Adolf Krige, Kaspar Valgepea, Oskar Modin, Yvonne Nygård, Carl Johan Franzén (2025). “Heterologous expression of pyruvate formate lyase in *Clostridium ljungdahlii* enhances cell growth in a bioelectrochemical system”. (*manuscript*)

## **Contribution Summary**

My contributions to Papers I-IV were as follows:

- I. Took part in conceptualization. Performed the experimental work, analysis of the data, the writing, and editing of the manuscript.
- II. Took part in conceptualization. Performed the experimental work, analysis of the data, the writing, and editing of the manuscript.
- III. Took part in Took part in conceptualization. Performed the experimental work, formal analysis, the writing, and editing of the manuscript.
- IV. Conceptualized and planned the study. Performed the experimental work, analysis of the data, the writing, and editing of the manuscript.

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## Abbreviations

ALE	Adaptive laboratory evolution
BES	Bioelectrochemical system
HER	Hydrogen evolution reaction
MFC	Microbial fuel cell
SHE	Standard hydrogen electrode
PFL	Pyruvate formate lyase



## Chapter 1. Introduction

---

Methane ( $\text{CH}_4$ ), carbon monoxide ( $\text{CO}$ ), and carbon dioxide ( $\text{CO}_2$ ) are highly abundant greenhouse gases known for their harmful effect on the environment, but also potential applications. Methane is a major product of biogas fermentation by methanogens, as well as a product of shale gas and natural gas extraction. The prolonged exploitation of fossil fuels has resulted in the establishment of infrastructure for methane utilization. It has also led to the release of vast amounts of  $\text{CO}_2$  and  $\text{CO}$ , which are two main contributors to global warming. Therefore, in this thesis, I will focus on the utilization of  $\text{CO}_2$  and  $\text{CO}$ .

### 1.1. $\text{CO}$ and $\text{CO}_2$ : pollutants or raw materials?

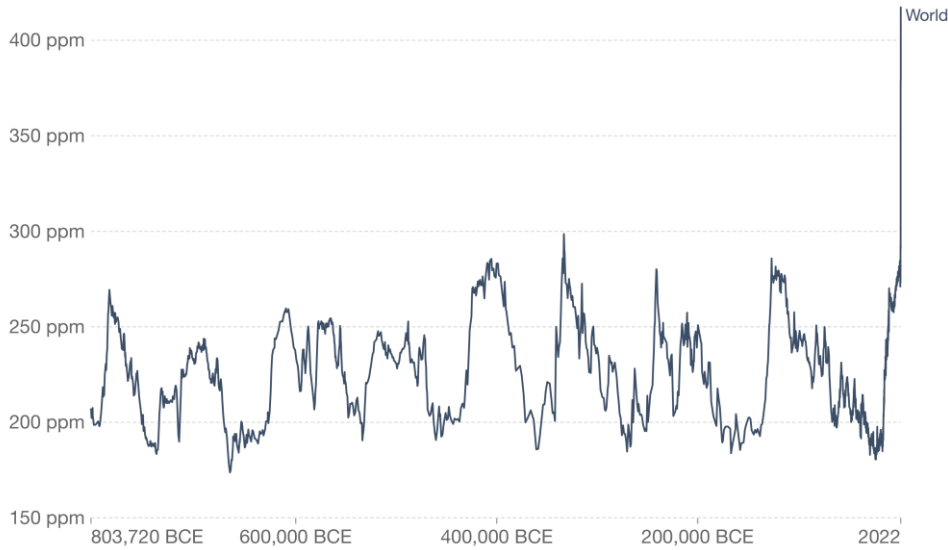
$\text{CO}_2$  is a known greenhouse gas produced from the complete combustion of carbon-based materials, such as fossil fuels and lignocellulosic biomass. Consequently,  $\text{CO}_2$  is commonly emitted in industrial processes, power plants that burn fossil fuels, and transportation. Since the Industrial Revolution enabled mass production, the demand for electricity has increased significantly. As a result, fossil fuel use for heat generation has grown alongside technological advancements. While humanity has achieved an unprecedented degree of prosperity, the same is true for the alarmingly high atmospheric  $\text{CO}_2$  levels (**Figures 1 and 2**).

$\text{CO}$  is produced from the incomplete combustion of carbon-based materials. While  $\text{CO}$  has no direct environmental impact, it is flammable, toxic, and an indirect risk to the atmosphere [1].

Emissions of  $\text{CO}$  and  $\text{CO}_2$  have a negative impact on the environment, both directly and indirectly. However, they can be repurposed also as fuel sources. A gas mixture of  $\text{CO}$  and hydrogen ( $\text{H}_2$ ), known as synthesis gas (syngas), often also contains  $\text{CO}_2$  and  $\text{CH}_4$ , along with trace gases, such as nitrogen, ammonia, and hydrogen sulfide. Historically, syngas has been obtained from wood biomass and coal — hence it was previously called wood gas or coal gas — and used to fuel automobiles equipped with gasifiers. These vehicles were widely used during World War II. Later, some vehicles began using synthetic fuel produced through a process patented by Franz Fischer and Hans Tropsch [2]. Since then, syngas has gained significant attention as an alternative fuel source to meet the growing demand for liquid fuels.

### Global atmospheric CO<sub>2</sub> concentration

Atmospheric carbon dioxide (CO<sub>2</sub>) concentration is measured in parts per million (ppm). Long-term trends in CO<sub>2</sub> concentrations can be measured at high-resolution using preserved air samples from ice cores.



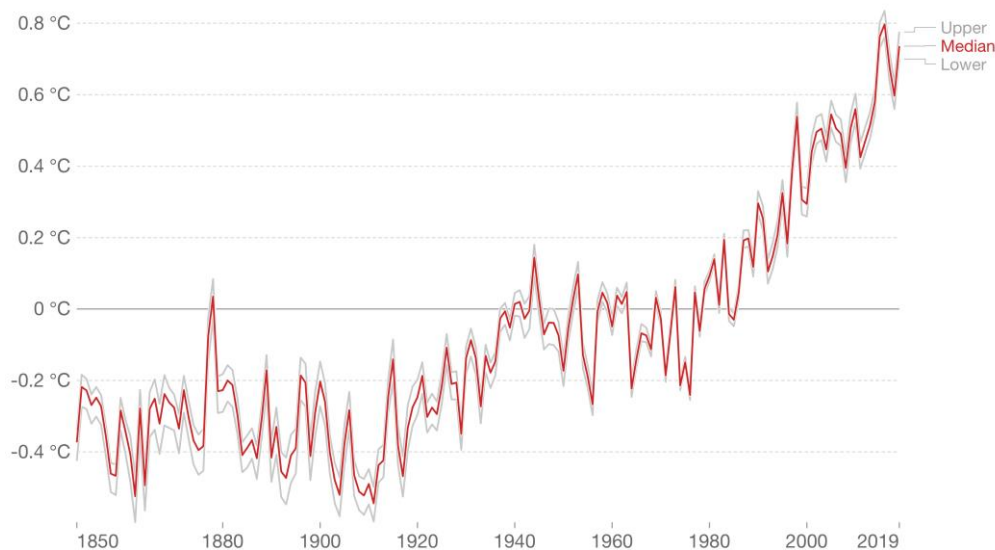
Source: National Oceanic and Atmospheric Administration (NOAA)

CC BY

**Figure 1 Global atmospheric CO<sub>2</sub> concentration.** Data were originated by the National Oceanic and Atmospheric Administration. Figure taken from Our World in Data (<https://ourworldindata.org/>).

### Average temperature anomaly, Global

Global average land-sea temperature anomaly relative to the 1961-1990 average temperature.



Source: Hadley Centre (HadCRUT4)

OurWorldInData.org/co2-and-other-greenhouse-gas-emissions • CC BY

Note: The red line represents the median average temperature change, and grey lines represent the upper and lower 95% confidence intervals.

**Figure 2 Global average temperature anomaly.** Data were originated by the Hadley Centre. Figure taken from Our World in Data (<https://ourworldindata.org/>).



Currently, additional sources of syngas include biowaste and steel mill off-gas [3]. However, the Fischer-Tropsch process requires high temperature (200–400 °C), high pressure (2–5 MPa), and a specific H<sub>2</sub>/CO ratio [2, 4]. Furthermore, it has intrinsic problems resulting from the use of metal catalysts, such as low yield due to CO methanation and the water-gas shift reaction. The latter causes deactivation of catalysts by water produced during the process, along with coke formation [4]. Therefore, a more resilient and flexible catalyst is required for syngas conversion into synthetic liquid fuel.

## 1.2. Gas fermentation

Since ancient times, fermentation has represented a traditional means of food production and preservation using autochthonous microorganisms [5]. The invention of the microscope and advancements in biotechnology have enabled the study and widespread application of microorganisms [6]. Existing tools allow us to engineer microorganisms' genetic traits and apply fermentation for the industrial manufacturing of commodity chemicals in a process known as a biorefinery [7].

Biorefineries utilize microbial cell factories to produce fuels and chemicals in an ecofriendly manner, often combined with thermochemical, chemical, and enzymatic conversions [8]. Microbial fermentation is typically performed at medium temperature (28–38 °C) and ambient pressure, making it less energy-intensive and safer than chemical processes. The concept of a biorefinery has been developed based on the choice of feedstock. First-generation biorefineries use starch- and sugar-containing food biomass, such as corn, soy, and sugarcane, which can lead to food shortages and an increase in food prices. Second-generation biorefineries employ non-edible biomass as feedstock, however, even this implies the diversion of arable land from food crops. CO<sub>2</sub> is an emerging, third-generation feedstock for biorefineries [9]. However, because CO<sub>2</sub> is the most oxidized form of carbon, additional reducing equivalents are required to convert CO<sub>2</sub> into value-added chemicals.

Biological CO<sub>2</sub> reduction and H<sub>2</sub> oxidation for acetate production by acetogenic bacteria (acetogens) was first reported in 1932. Later, biological utilization of syngas as feedstock by acetogens for chemical and fuel production was suggested [10]. Using acetogens as biological catalysts for syngas conversion can overcome many challenges associated with chemical catalysts. Most acetogens are mesophilic (i.e., they live at 30–37 °C), thrive at flexible CO:CO<sub>2</sub>:H<sub>2</sub> substrate ratios, and produce specific compounds (**Table 1**) [11–13]. Moreover, biological catalysts are self-replicating by nature, avoiding concerns about catalyst deactivation or poisoning. However, the cultivation of microorganisms can also be challenging.

**Table 1. Characteristics of some acetogenic bacteria**

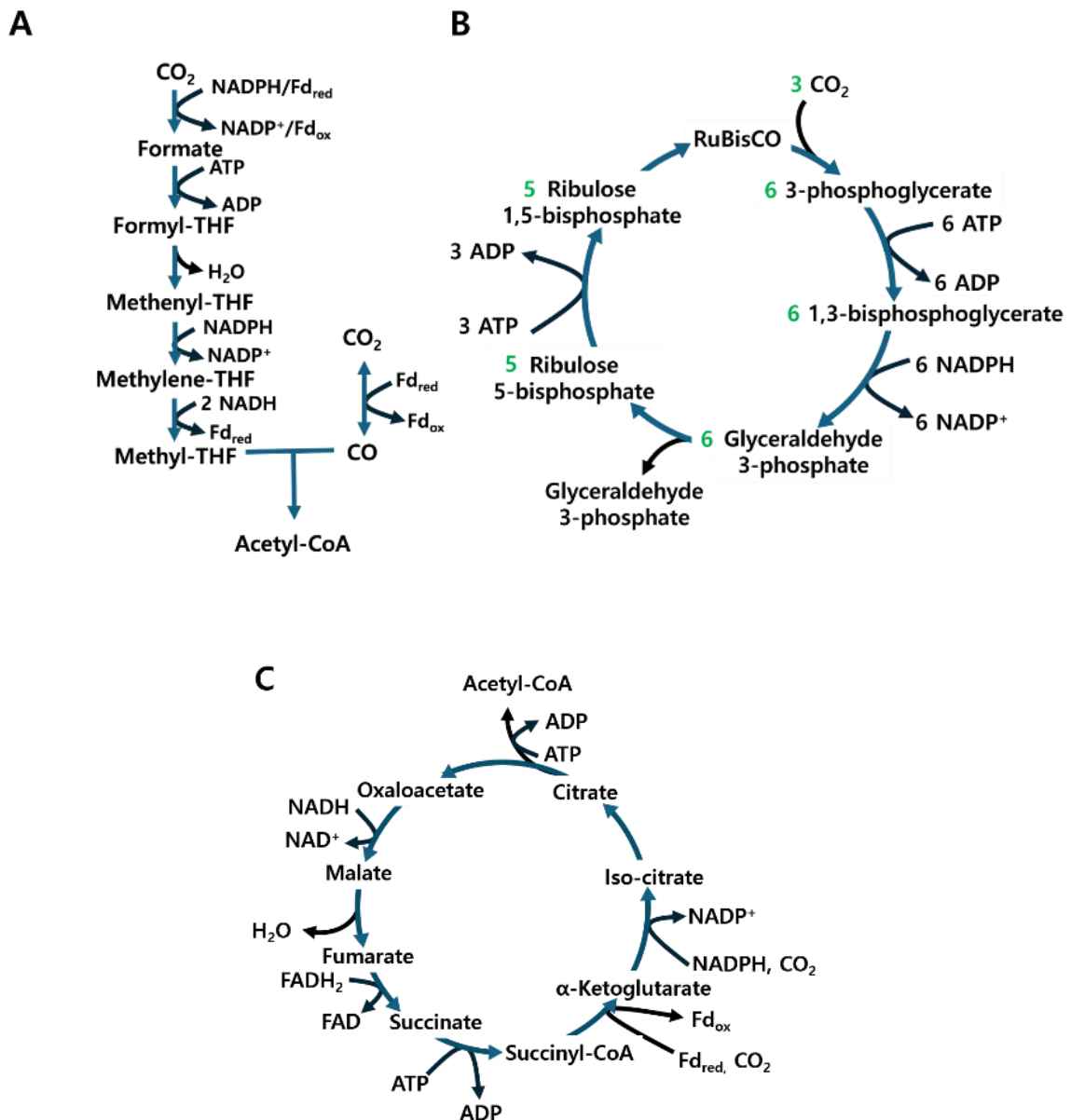
Acetogen	Substrates	Product(s)	Temp.	pH	Genome seq. avail.	Electro-activity	Ref
<i>Acetobacterium woodii</i>	H <sub>2</sub> /CO <sub>2</sub> (+ CO) <sup>1</sup>	Acetate	30	5.5-7.5	Yes	Yes, Mixed culture	[14, 15]
<i>Alkalibaculum bacchi</i>	H <sub>2</sub> /CO <sub>2</sub> , CO	Acetate, ethanol	37	8.0-8.5	Scaffold	Mixed culture	[16]
<i>Butyribacterium methylotrophicum</i>	H <sub>2</sub> /CO <sub>2</sub> , CO	Acetate, ethanol, butyrate, butanol	37	6.0			[17]
<i>Clostridium autoethanogenum</i>	H <sub>2</sub> /CO <sub>2</sub> , CO	Acetate, ethanol, 2,3-BD, lactate	37	4.5-6.0	Yes	Yes <sup>2</sup> , Mixed culture	[18]
<i>Clostridium carboxidivorans (P7)</i>	H <sub>2</sub> /CO <sub>2</sub> , CO	Acetate, ethanol, butyrate, butanol, lactate	38	5.0-7.0	Yes		[19]
<i>Clostridium coskatii</i>	H <sub>2</sub> /CO <sub>2</sub> , CO	Acetate, ethanol	37	5.8-6.5	Contig		[20]
<i>Clostridium drakei</i>	H <sub>2</sub> /CO <sub>2</sub> , CO	Acetate, ethanol, butyrate	30	3.6-7.4			[21]
<i>Clostridium ljungdahlii</i>	H <sub>2</sub> /CO <sub>2</sub> , CO	Acetate, ethanol, 2,3-BD, lactate	37	4.5-7.0	Yes	Yes	[22, 23]
<i>Clostridium ragsdalei (P11)</i>	H <sub>2</sub> /CO <sub>2</sub> , CO	Acetate, ethanol, 2,3-BD, lactate	37	6.3	Contig		[24]
<i>Clostridium scatologenes</i>	H <sub>2</sub> /CO <sub>2</sub> , CO	Acetate, ethanol, butyrate	37	3.9-7.0	Yes		[25]
<i>Eubacterium limosum</i>	H <sub>2</sub> /CO <sub>2</sub> , CO	Acetate, butyrate	38	6.7-7.2	Yes	Mixed culture	[26, 27]
<i>Moorella thermoacetica</i>	H <sub>2</sub> /CO <sub>2</sub> , CO	Acetate	55	6.5-7.0	Yes		[28]
<i>Moorella thermoautotrophica</i>	H <sub>2</sub> /CO <sub>2</sub> , CO	Acetate	58	6.1		Yes	[29]
<i>Thermoanaerobacter kivui</i>	H <sub>2</sub> /CO <sub>2</sub> , CO	Acetate	66	7.0-7.5			[30]

<sup>1</sup> The wild-type strain cannot grow on CO as the sole carbon and electron source

<sup>2</sup> Not tested with autotrophic conditions

### 1.2.1. Metabolic pathways and products

There are three natural metabolic pathways for CO<sub>2</sub> assimilation: the Wood-Ljungdahl pathway, the Calvin-Benson-Bassham cycle, and the reductive tricarboxylic acid (TCA) cycle [31] (**Figure 3**). The Calvin-Benson-Bassham cycle and the reductive TCA cycle are energetically inefficient, requiring additional ATP sources (**Table 2**).



**Figure 3. Schematic diagram of three well-known bacterial metabolic pathways for CO<sub>2</sub> assimilation.** (A) Wood-Ljungdahl pathway, (B) Calvin-Benson-Bassham cycle, and (C) reductive TCA cycle

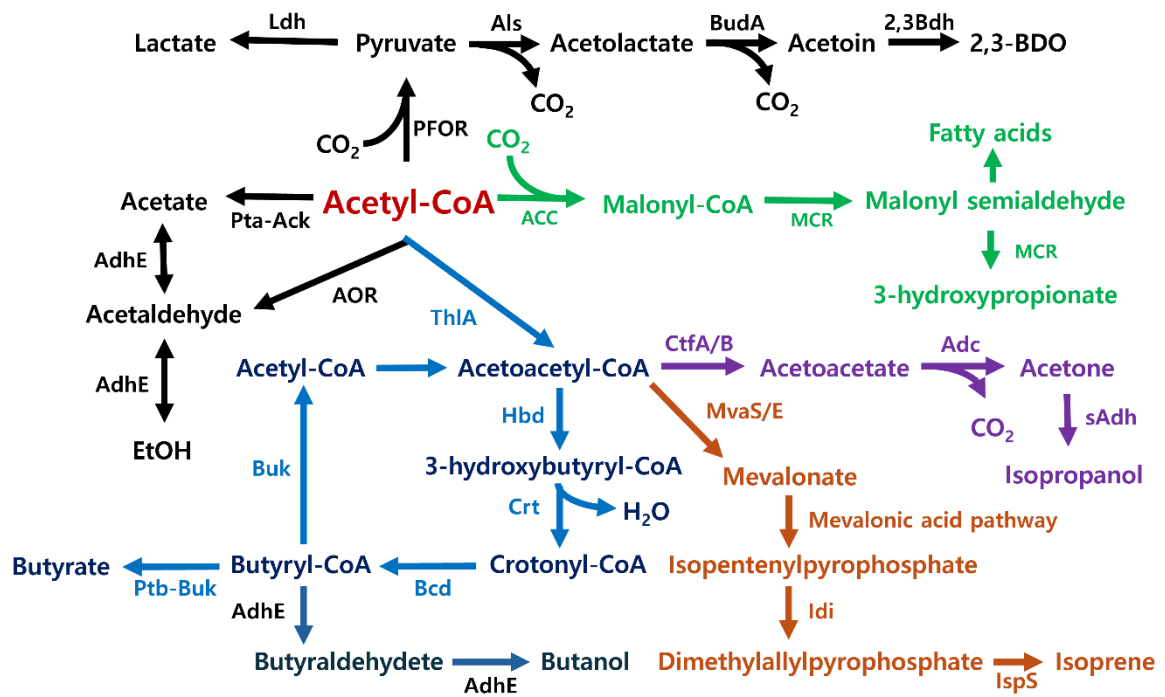
**Table. 2 Comparison required ATP and NAD(P)H of CO<sub>2</sub> assimilation pathways**

Pathway	The number of CO <sub>2</sub> assimilated	ATP required	NAD(P)H (Fd <sub>red</sub> ) required	Product
Wood-Ljungdahl pathway	2	1	3 (1)	Acetyl-CoA (C2)
Calvin-Benson-Bassham cycle	3	9	6	Glyceraldehyde 3-phosphate (C3)
Reductive TCA cycle	2	2	3 (1)	Acetyl-CoA (C2)

Therefore, microorganisms that rely on these pathways exhibit slow growth, as well as low product and biomass yield. The Wood-Ljungdahl pathway is the most efficient means of assimilating single-carbon molecules, generating extra ATP *via* acetate kinase (Ack) and *via* ATP synthase, and consuming a wide range of substrates, including CO, CO<sub>2</sub>, formate, and methanol [32]. Therefore, gas fermentation relies on acetogens equipped with the Wood-Ljungdahl pathway. The main product of the Wood-Ljungdahl pathway is acetyl-CoA. Thus, acetyl-CoA derived chemicals, such as acetate, ethanol or CH<sub>4</sub> (if methanogens are used) are the typical target products. Some acetogens produce also lactate, 2,3-butanediol, butyrate, hexanoate, and their corresponding alcohols with the help of the reverse  $\beta$ -oxidation pathway (**Figure 4**). Additionally, newly developed genetic tools make it possible to expand the range of products to include acetone, isopropanol, and 3-hydroxypropionic acid [13].

### 1.2.2. Host strains

Since isolation of the first acetogen, *Clostridium aceticum* [33, 34], a variety of other candidates have emerged [35]. The most extensively studied acetogens for industrial applications are *Acetobacterium woodii*, *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, *Clostridium carboxidivorans*, *Eubacterium limosum*, *Moorella thermoacetica*, and *Thermoanaerobacter kivui*. Each acetogen has a different substrate preference, product spectrum, optimal growth pH, and temperature (**Table 1**). Therefore, the choice of a host strain should be based on its target application, feedstock used, and desired products.



**Figure 4. Schematic diagram of metabolic pathways that start from acetyl-CoA.** Black lines denote naturally occurring metabolic pathways in *Clostridium ljungdahlii*. Green lines denote the malonyl-CoA pathway for 3-hydroxypropionate and fatty acids synthesis. Blue lines denote the reverse  $\beta$ -oxidation pathway. Purple lines denote the acetone production pathway. Orange lines denote the isoprene production pathway. 2,3 Bdh, 2,3-butanediol dehydrogenase; ACC, acetyl-CoA carboxylase; Ack, acetate kinase; Adc, acetoacetate decarboxylase; AdhE, aldehyde/alcohol dehydrogenase; Als, acetolactate synthase; AOR, aldehyde:ferredoxin oxidoreductase; Bcd, butyryl-CoA dehydrogenase; BudA, acetoin decarboxylase; Buk, butyrate kinase; Crt, crotonase; CtfA/B, acetoacetyl-CoA:acetate/butyrate-CoA transferase; Hbd, 3-hydroxybutyryl-CoA dehydrogenase; Idi, isopentenyl pyrophosphate isomerase; IspS, isoprene synthase; Ldh, lactate dehydrogenase; MCR, malonyl-CoA reductase; MvaE, hydroxymethylglutaryl-CoA reductase; MvaS, hydroxymethylglutaryl-CoA synthase; PFOR, pyruvate:ferredoxin oxidoreductase; Pta, phosphotransacetylase; Ptb, phosphotransbutyrylase; sAdh, secondary alcohol dehydrogenase; ThIA, thiolase;

### 1.2.3. Processes

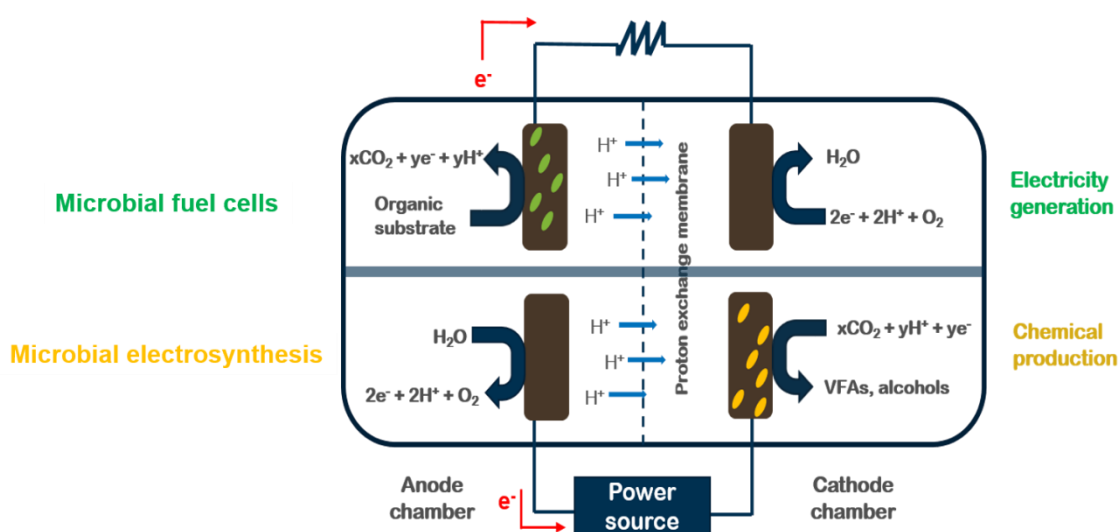
The fermentation process should be selected based on the type of feedstock and target products. The development of gas fermentation processes has focused primarily on improving gas-liquid mass transfer to overcome the low solubility of CO and H<sub>2</sub> [36]. As a result, various reactor types have been introduced for gas fermentation, including continuous stirred-tank reactors, bubble column reactors, trickle bed reactors, hollow fiber membrane reactors, and gas-lift reactors [36]. At the same time, bioelectrochemical systems (BESs) for

CO<sub>2</sub> and CO fermentation have been introduced to provide additional reducing equivalents [37, 38].

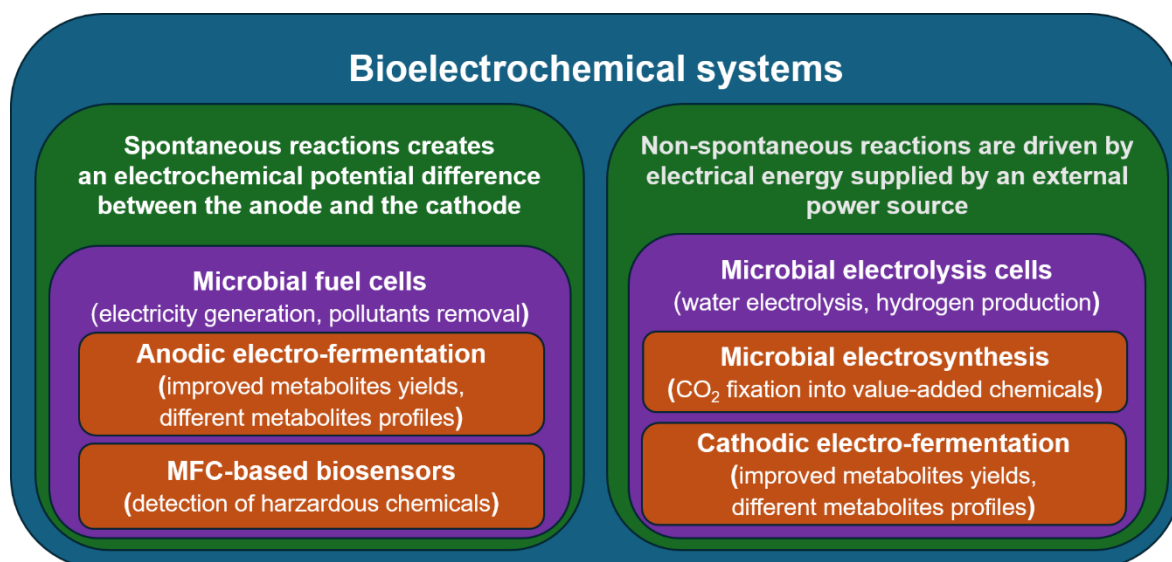
### 1.3. Bioelectrochemical systems

Many biological reactions are catalyzed by redox enzymes. These redox reactions often involve endogenous electron carriers, such as NAD(P)H, FADH<sub>2</sub>, ferredoxin, and ubiquinone. The redox balance plays an important role in cell metabolism and metabolite production. Some bacteria can interact with each other and/or with the environment to regulate their intracellular redox balance [39]. Dissimilatory metal-reducing bacteria capable of oxidizing organic carbon, leading to ecologically significant carbon mineralization, have also been reported [40, 41]. Notably, these bacteria reduced not only metals, but also electrodes in electrochemical cells, providing the first demonstration of a microbial fuel cell (MFC) that did not require an exogenous electron mediator [40, 42]. Dissimilatory metal- or electrode-reducing processes enable anaerobic bacteria to run the TCA cycle in what is termed anaerobic respiration. As a result, bacteria involved in bioelectrochemical applications are classified as electro-active bacteria [43].

A typical BES reactor setup consists of a cathode and an anode chamber separated by a separator (e.g., a proton exchange membrane) (**Figure 5**). The cathode and the anode are connected by an external electric circuit to facilitate current flow. Electro-active bacteria can be introduced either to the cathode or anode chamber of a BES, giving rise to different classes of BES (**Figure 6**).



**Figure 5.** Schematic diagram of bioelectrochemical systems.



**Figure 6. Classification of bioelectrochemical systems.**

### 1.3.1. Microbial fuel cells

A MFC is a type of BES that converts chemical energy into electrical energy, generating electricity (**Figure 5 – 6**). In the anode chamber, electro-active bacteria oxidize organic substrates and transfer electrons to the anode under anaerobic conditions. Electron acceptors with a higher standard redox potential than the anode reduction reaction (e.g., oxygen, ferricyanide) are present in the cathode chamber, creating an electrochemical potential difference between the anode and cathode and enabling the flow of electric current [44]. A separator prevents bacteria and electron acceptors from crossing between the chambers, while facilitating proton exchange to balance the pH difference across them and, thus, ensure a sustainable operation. Electrons from the anode are transferred through a circuit, which consumes electrical power (e.g., resistors or electrical devices). Isolation of *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1 enabled the development of mediator-less MFCs [40-42, 45]. The type strains *S. oneidensis* MR-1 and *G. sulfurreducens* PCA have revealed how electro-active bacteria exchange electrons with solid-state electrodes and metals, as well as how extracellular electron transfer by these bacteria is closely related to their metabolism [39, 43]. This and other studies have enabled the engineering of microorganisms with enhanced electro-activity [46, 47].

Since its discovery by Michael Potter [48], microbial electricity generation has found notable practical application in wastewater treatment [49]. Replacing the aeration process for biological degradation of dissolved organic pollutants in a wastewater treatment plant with a MFC that relies on anaerobic respiration halves the operational costs [50]. Therefore, MFCs can conveniently generate bioelectricity while simultaneously treating wastewater and removing pollutants. Additionally, they can be used for H<sub>2</sub> production in microbial

electrolysis cells (by employing protons as electron acceptors at the cathode with an external power input), as power supply for environmental sensors, for nutrient recovery, as biosensor for pollutants, and for hazardous chemical detection [51].

### 1.3.2. Electro-fermentation

While MFCs gain attention as an innovative biotechnological application, electro-fermentation is attracting interest as a potential breakthrough to overcome the limitations of traditional fermentation [52]. The latter includes redox imbalances that restrict efficiency and often imply additional costs for co-substrate or gas-sparging with H<sub>2</sub>, O<sub>2</sub> or air [53]. In contrast to genetic engineering as a means to improve performance, electro-fermentation leverages BESs to regulate the intracellular redox balance, facilitate product selectivity, and boost yields [52, 54].

Evaluating the efficiency of electro-fermentation requires assessing multiple aspects of its performance, including metabolic output, energy input, and system stability. Key parameters, such as product yield, selectivity, and volumetric productivity, are as critical in electro-fermentation as in traditional fermentation. Additionally, energy and coulombic efficiency must be specifically evaluated in electro-fermentation to account for the impact of the BES. Coulombic efficiency in electro-fermentation is calculated by dividing the total number of electrons transferred to the products by the total number of electrons supplied by the electrode. This indicates how efficiently the supplied electrons are utilized for product formation (**Equation 1**).

$$CE(\%) = \frac{\text{Total number of electrons recovered in products}}{\text{Total number of electrons supplied by the electrode}} \times 100\% \quad \text{(Equation 1)}$$

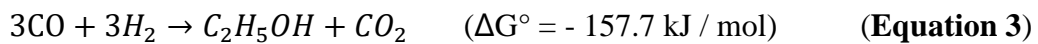
Initially, electro-fermentation was tested with exogenous electron mediators [55, 56]. Later, *G. sulfurreducens* and *Geobacter metallireducens* were found to take up electrons straight from the cathode [57], paving the way for direct electron uptake-driven application of bacterial metabolism [58]. At the same time, the use of H<sub>2</sub> produced at the cathode by methanogens in a microbial electrolysis cell was first suggested and then experimentally demonstrated [59, 60]. Importantly, CO<sub>2</sub> fixation offers the possibility to store electrical energy in the form of chemical energy in a BES, leading to microbial electrosynthesis of organic compounds using acetogenesis from CO<sub>2</sub> [61, 62].

### 1.3.3. CO electro-fermentation

Microbial electrosynthesis offers a carbon capture and utilization mechanism for chemical production using renewable energy [63]. At present, the applicability of microbial electrosynthesis is limited by the low CO<sub>2</sub> content of industrial waste gas or the elevated



costs of CO<sub>2</sub> purification [37, 64]. However, CO-containing waste gases are easily found or easily obtained from various sources such as syngas [3]. Gas fermentation technology using acetogens and syngas is mature enough to be commercially viable and could potentially replace at least part of the petrochemical-based manufacturing, as illustrated by LanzaTech [65]. When gas fermentation uses CO-rich gas, substantially more H<sub>2</sub> is required to fix the CO<sub>2</sub> present in syngas or generated via CO oxidation by CO dehydrogenase (**Equations 2–5**) [11].

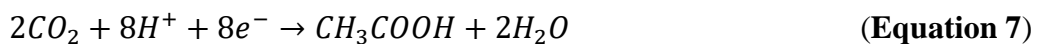


The first study about CO conversion in a BES using a mixed culture as the inoculum sought to increase carbon recovery from CO-containing waste gas [37]. The study achieved >200% coulombic efficiency based on the ratio of electrons recovered in the metabolites produced to the electrons converted from the current flow. This finding indicated that the BES facilitated re-assimilation of CO<sub>2</sub> released during CO fermentation (**Equation 6 – 8**) [37].

Acetate production by CO-oxidizing bacteria:



Acetate production by CO<sub>2</sub> re-assimilation in a BES:

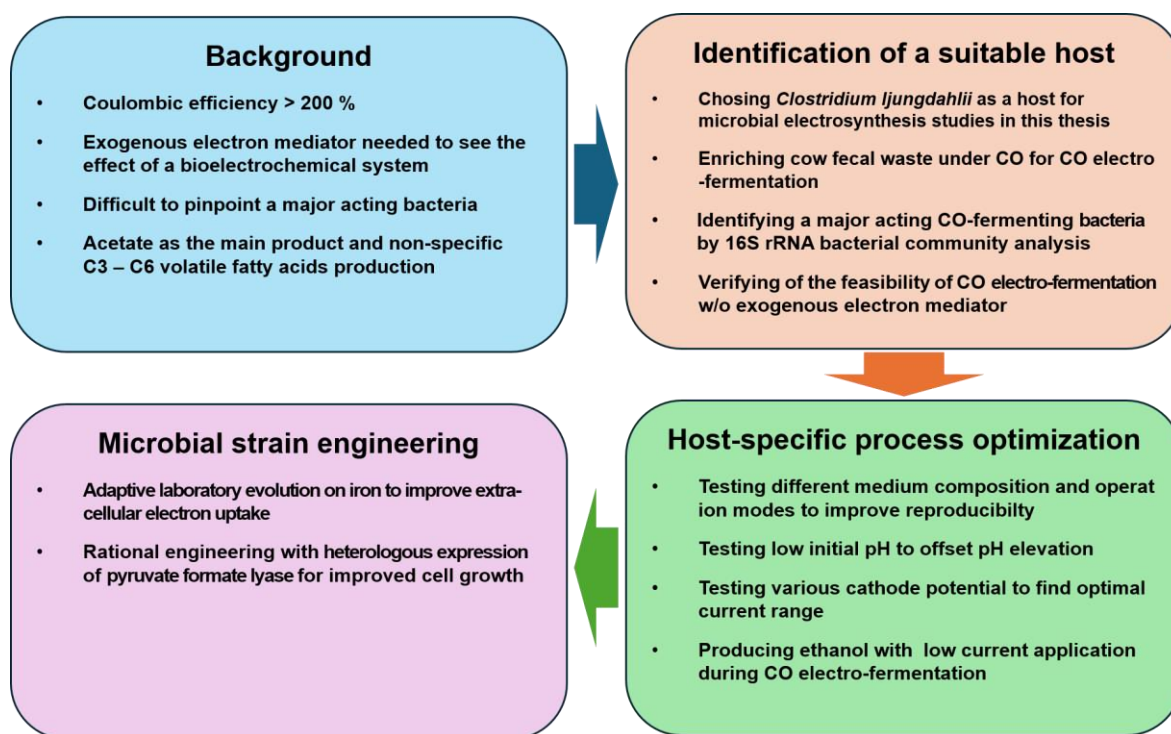


Hypothesized total reaction by CO electro-fermentation (**Equation 6 + Equation 7**):



## 1.4. Aims and scope

The overall goal of this thesis was to improve the performance of CO electro-fermentation for the production of ethanol. In spite of earlier reports on CO electro-fermentation [37, 66–69], its applications remain limited due to various factors. These include the 1) requirement of exogenous electron mediator for extracellular electron uptake, which increases operational costs, is difficult to recycle, and might be toxic to the environment, for CO-fermenting bacterial communities from mixed cultures, 2) non-specific target products (**Chapter 2**), 3) unclear extracellular electron uptake mechanism (**Chapter 3**), and 4) lack



**Figure 7. Workflow and main activities for the development of CO electro-fermentation as an example.**

of attempts to metabolically engineer acetogens for improved performance in microbial electrosynthesis (**Chapter 4**). The feasibility of CO electro-fermentation will be discussed after addressing the aforementioned challenges and unresolved questions (**Chapter 5**).

The aims of this thesis were to identify a major gas-fermenting bacterium for CO electro-fermentation (**Paper I**) and enhance its performance. The latter was achieved by optimizing operational parameters for a BES (**Papers I and II**), along with evolutionary (**Paper III**) and rational (**Paper IV**) strain engineering (**Figure 7**).

In this thesis, these aims were addressed in the following ways:

**Identifying a suitable host strain for CO electro-fermentation** was done by enrichment of cow fecal waste under CO conditions without the addition of an exogenous electron mediator. The bacterial community in the CO-enriched mixed culture was analyzed by metagenomic 16S rRNA sequencing and tested for electro-activity in a BES and CO electro-fermentation (**Paper I**). This result confirmed the appropriateness of selecting *C. ljungdahlii* as a host strain for CO electro-fermentation.

**Host-specific process optimization** was done by testing various cathode potentials (or applying different electric current), different initial pH, and culture media both with and without yeast extract supplementation (**Papers I and II**).

**Microbial strain engineering for improved acetogenic performance in a BES** employed both evolutionary (**Paper III**) and rational (**Paper IV**) approaches.

- Evolutionary engineering employed iron as the sole electron donor during adaptive laboratory evolution (ALE) to cultivate *C. ljungdahlii* for the improvement of extracellular electron uptake in a BES in **Paper III**, since iron oxidation is akin to the reduction reaction at the cathode in a BES.
- Rational engineering addressed the challenge of poor growth of *C. ljungdahlii* by introducing an alternative route for pyruvate synthesis, which is the first step of gluconeogenesis, with formate as a co-substrate.

Hence, in this thesis, **Chapter 2** provides a broad overview of CO electro-fermentation for host strain selection, along with a description of *C. ljungdahlii*, which is the host strain used in this thesis, and acetogenic bacterial communities operated in **Paper I**. Additionally, the chapter discusses key factors for development and optimization of CO electro-fermentation processes emerged from **Papers I** and **II**. **Chapter 3** explores extracellular electron transfer mechanisms and investigates potential extracellular electron uptake mechanisms in acetogens. It further discusses strategies to enhance such extracellular electron uptake and highlights how ALE can be employed to achieve this goal, as attempted in **Paper III**. **Chapter 4** provides a brief overview of techniques for the genetic engineering of acetogens, before suggesting potential strategies for improving the performance of CO electro-fermentation and presenting findings from **Paper IV**. Finally, **Chapter 5** summarizes the findings and suggests appropriate strategies for the realization of CO electro-fermentation.



## Chapter 2. CO electro-fermentation

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This chapter focuses on the criteria and procedure for host strain selection, as well as development and optimization of CO electro-fermentation. Host strain selection is a critical step in the development of fermentation processes. For microbial electrosynthesis or CO electro-fermentation to achieve economic viability, the resulting metabolites must have significant market potential. Industrially relevant acetogenic strains capable of solvent production are promising candidates for this purpose. The selection or development of a high-performing acetogenic strain should be optimized specifically for use in a BES reactor. Integrating gas fermentation and electro-fermentation with a poorly performing acetogen would be inefficient and lack practical value.

In addition, optimization of the fermentation process ensures that the host strain performs at its full potential. **Section 2.1** introduces important operational parameters for microbial electrosynthesis and CO electro-fermentation, drawing on the findings from **Papers I, II, III, and IV**. It also addresses reactor design and process development, which are critical for biotechnological applications, as they provide a controlled and ideal environment for microbial cells to grow and carry out their metabolic processes.

**Section 2.2** outlines the criteria used in selecting strains for microbial electrosynthesis and CO electro-fermentation, and reviews previous studies alongside the operational parameters discussed in **section 2.1**. This chapter introduces the host strain used in this thesis work, *C. ljungdahlii*, and highlights its relevance and potential for the processes proposed herein. Also, Metagenomic 16S rRNA bacterial community analysis of CO-enriched mixed culture confirms the major acting bacteria in CO electro-fermentation.

### 2.1. Process development and optimization

Electro-fermentation integrates an electrochemical system with a fermentation reactor to regulate the intracellular redox balance and produce large amounts of target products (**Papers I, II, III, and IV**) [53]. Consequently, additional electrochemical operational parameters, such as electrode potentials, medium conductivity, and the effects of current flow, must be carefully considered. Most importantly, integration of an electrochemical system should create synergistic benefits rather than adverse effects. **Section 2.1.1** considers

the operational parameters relevant for electro-fermentation and how they affect performance (**Papers I, II, III, and IV**).

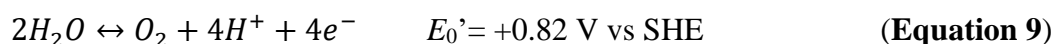
The **design of a fermentation reactor** is crucial because it directly impacts the efficiency, scalability, and quality of the biological processes inside. A well-designed fermentation reactor provides adequate conditions for microbial cells to produce the desired products, while also addressing economic and operational challenges. Key elements for electro-fermentation, such as positioning of electrodes and separators between the anode and cathode chambers of a BES, will be discussed in **section 2.1.2**.

### 2.1.1. Operational parameters and culture conditions

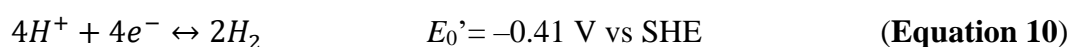
Electro-fermentation is conducted in a bioreactor with electrodes connected to an external power source. The key operational parameters are electrode potentials and electric current. Typically, the working electrode potentials are set based on the standard redox potential of the target reaction. The electric current flow represents the number of electrons involved in electrochemical reactions. The driving force for an electrochemical reaction is the energy difference between the electrons in the electrode and the energy level of the reactants or products involved in the redox reaction, typically represented by their electrochemical potentials [70]. This driving force increases as the difference between the electrode potential and the redox potential of the reaction grows, which accelerates the rate of the electrochemical reaction [71]. However, for large-scale electro-fermentation, several other factors affecting this potential difference, such as electrolyte pH and sustained cell performance, must be carefully considered (**Papers I and II**).

Reduction reactions do not always require protons, but many do as part of the overall process. Electro-fermentation relies on an aqueous environment for bacterial cultivation. In aqueous or acidic conditions, the hydrogen evolution reaction (HER), also known as the proton reduction reaction on the cathode, becomes favorable when water electrolysis is driven by an external power source.

On the anode:



On the cathode:

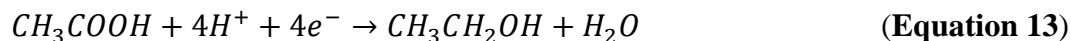
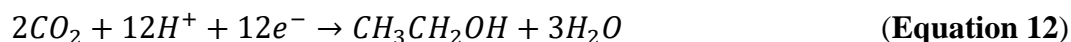
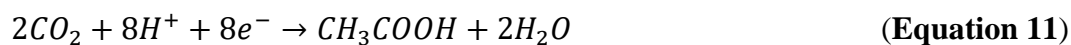


HER without an external power source  $\Delta G^{\circ} = +79.9 \text{ kJ/mol}$

HER at  $-0.5 \text{ V}$  electrode potential  $\Delta G^{\circ} = -16.6 \text{ kJ/mol}$

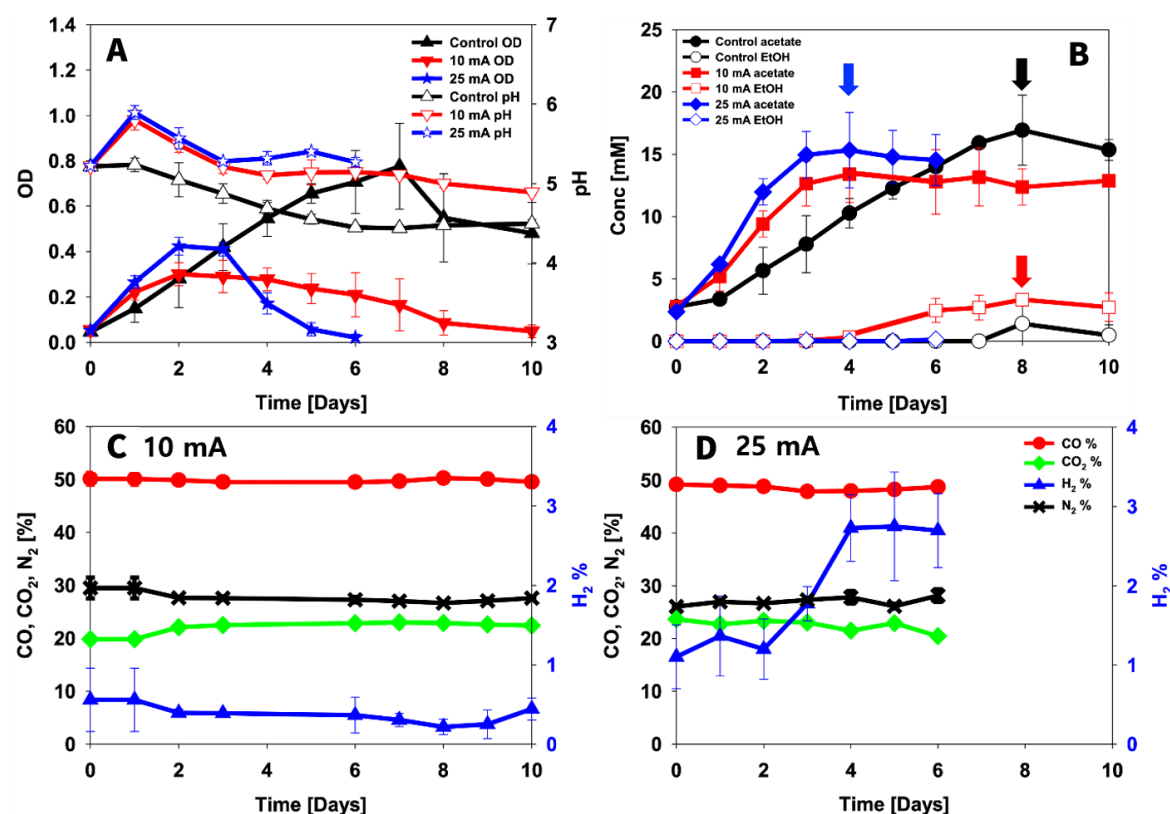
HER at  $-1.0$  V electrode potential  $\Delta G^\circ = -113.07$  kJ/mol

Many electro-fermentation reactions involve consumption of protons to produce metabolites:



Therefore, protons in the medium are quickly consumed by the reduction reaction. While water electrolysis at the anode can supply protons through the proton exchange membrane, their mobility is typically slower than the rate of reduction at the cathode [72, 73]. Eventually, this alters the pH in the cathode chamber, which may constrain the growth of acetogens and the rate of microbial electrosynthesis (**Papers I and II**). This limitation becomes even more pronounced in biofilm-driven microbial electrosynthesis [74, 75]. Conversely, some reactions such as ethanol production are more favorable at mildly acidic pH ( $\sim 5.0$ ) [76]. In either case, high electric current flow with lower energy input is required to have scalable electro-fermentation technology [32]. A highly saline medium was tested to increase conductivity, but acetate was the main product [77]. Therefore, pH and cathode potential (or current density) need to be carefully tuned, as was done in **Papers I and II** for CO electro-fermentation and microbial electrosynthesis.

Two different electric currents (10 and 25 mA) were tested for ethanol production via CO electro-fermentation using a mixed culture (**Paper I**). CO fermentations using industrially relevant acetogenic strains aim to produce ethanol due to its economic values and versatility [78]. However, CO electro-fermentation studies have not yielded any ethanol; only acetate and other volatile fatty acids (**Table 2** in **Paper I**). In this thesis, two strategies were employed to enable ethanol production during CO electro-fermentation. The first strategy involved the enrichment of solventogenic acetogens (**Section 2.2** and **Paper I**), while the second focused on optimizing the applied electric current. The two electric currents were selected based on the cathode potentials tested in **Paper II**. In that study, *C. ljungdahlii*, which is indistinguishable from *C. autoethanogenum* by 16S rRNA analysis and shares genetic similarity with it—the dominant species in the bacterial community used in **Paper I**—was used as a host for microbial electrosynthesis. Results from **Figure 4** in **Paper I** and **Figure 3** in **Paper II** show that high electric current (25 mA) or low cathode potential ( $-1.2$  V vs 3 M Ag/AgCl) increased the pH in the cathode chamber. A pH above the optimal pH for cell growth negatively affected both cell viability (**Figure 4** in **Paper I** and **Figure 3** in **Paper II**) and ethanol production. Poor cell viability resulted in lower coulombic efficiency than the one observed at low electric current (10 mA) or other cathode potentials tested

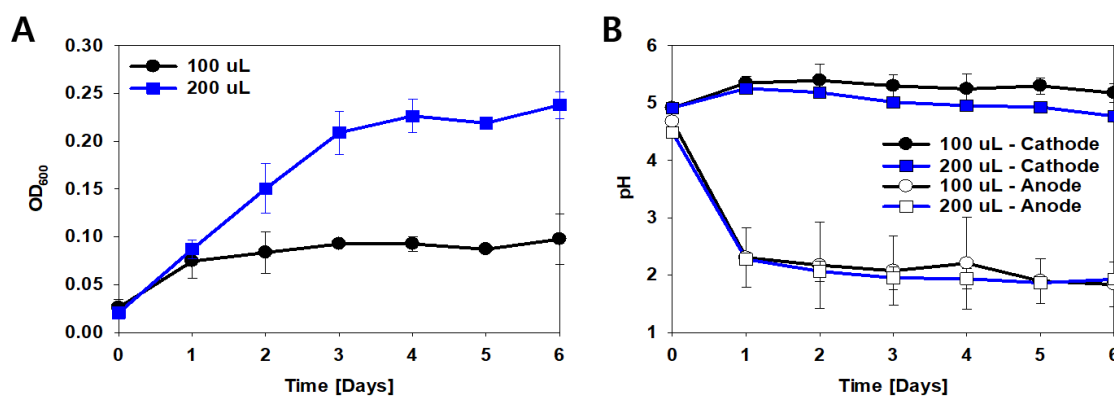


**Figure 8.** CO electro-fermentation using the enriched culture in H-type reactors sparged with 50% CO, 20% CO<sub>2</sub>, and 30% N<sub>2</sub> without addition of an exogenous electron mediator. (A) pH and optical density (OD); (B) metabolite concentrations; (C, D) headspace gas composition in reactors operated at (C) 10 mA and (D) 25 mA. Error bars denote the standard deviation (n = 3). Figure taken from **Paper I**.

(−0.6, −0.8, and −1.0 V vs 3 M Ag/AgCl) (**Papers I and II**). In fact, an elevated electric current is not essential for CO electro-fermentation in a BES (**Figure 8A, B**), because CO itself serves as an additional electron donor alongside the cathode.

Adjusting the initial pH of both the cathode and anode chambers of a BES reactor can improve microbial electrosynthesis performance (**Paper II**). When different initial pH values of the anode chamber were tested for CO electro-fermentation, both productivity and coulombic efficiency were improved at lower pH [66]. This can be explained by improved proton availability in the cathode chamber. Proton mobility through a proton exchange membrane depends on the pH gradient across the membrane. Insufficient proton transfer through the membrane due to elevated pH in the anode chamber may inhibit growth of *C. ljungdahlii*. When 200 μL of 1 M H<sub>2</sub>SO<sub>4</sub> was added to the anode chamber, *C. ljungdahlii* grew significantly better (**Figure 9A**) than what reported in **Figure 3** of **Paper II** upon addition of 100 μL 1 M H<sub>2</sub>SO<sub>4</sub>. Addition of 200 μL 1 M H<sub>2</sub>SO<sub>4</sub> to the anode chamber did not change much its pH (**Figure 9B**). The increase in pH in the cathode chamber can be compensated by starting at a lower pH (**Papers II and IV**). When an engineered *C. ljungdahlii*





**Figure 9. Growth of *C. ljungdahlii* in a BES with different acid addition.** A) OD and B) pH in the anode and cathode chambers with 100  $\mu\text{L}$  (black) and 200  $\mu\text{L}$  (blue) 1 M  $\text{H}_2\text{SO}_4$  added to the anode chamber. The cathode potential was  $-0.9$  V vs Ag/AgCl. Error bars denote the standard errors ( $n = 2$ ).

was tested in a BES with initial pH of 5.0, it performed better than if starting at pH 5.7 in the cathode chamber.

Reproducibility is another important feature for scaling up electro-fermentation technology (**Papers II and III**). Microbial electrosynthesis performance using mixed cultures varies over time or across batches. Pure cultures offer more consistent performance [79]. Nevertheless, even a pure culture subjected to identical operational conditions in a MES may face variations in current flow [80]. This variability is thought to arise from the complexity of electrochemical interactions and nutritional limitations under electron-limited conditions in a BES (**Supplementary information in Paper II and Figure 5 in Paper III**). To address this, in **Paper II**, *C. ljungdahlii* was tested both with or without yeast extract in the medium. The addition of yeast extract significantly improved the reproducibility of cell growth and metabolite production in MES. Similarly, application of a fixed electric current reduced the risk of inconsistent electric current flow, resulting in reproducible outcomes and improved growth of *C. ljungdahlii* in the BES, compared to application of a fixed cathode potential (**Figure 3 in Paper II and Figure 5 in Paper III**).

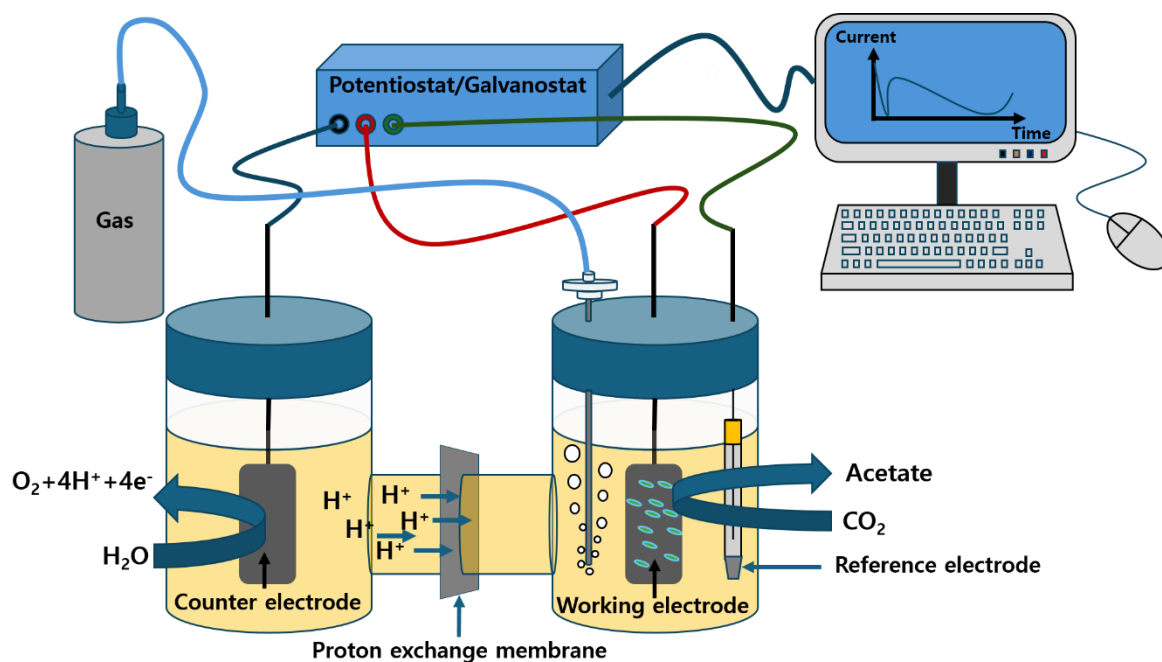
The impact of equilibrium constants on fermentation is not as direct as in purely chemical reactions, because biological systems are dynamic and involve multiple interacting factors. However, fermentation processes can still be influenced by equilibrium constants, particularly when enzymatic reactions within metabolic pathways approach equilibrium. For example, acetogenic output by *A. woodii* correlates with  $\text{H}_2$  partial pressure, highlighting how the latter is a key factor for the electron donor and, thus, a determinant of reaction efficiency [81]. The electric current in a BES reactor cannot reach levels comparable to those achieved with a gas composition of  $[80:20] = [\text{H}_2:\text{CO}_2]$ , which is commonly used for

cultivating autotrophic bacteria. Hence, a reactor design that suitably combines gas fermentation and electro-fermentation is crucial.

### 2.1.2. Reactor and process design

Many BES studies utilize an H-type reactor at the laboratory scale (**Figure 10**). Additionally, scalable electro-fermentation reactors have been developed to support industrial applications [82]. In a typical H-type BES reactor, the electrode is positioned in the middle of each chamber. An insufficient surface area of the working electrode can limit the efficiency of electro-fermentation. To address this issue, a nickel-phosphide-modified electrode has been tested to lower overpotential and facilitate H<sub>2</sub> production [83]. However, in large-scale electro-fermentation, uneven local electron supply poses a significant challenge, necessitating advanced reactor designs. To this end, the electrode can be integrated in the inner walls of the reactor, effectively increasing the surface area and ensuring a more uniform electron supply within the cathode chamber [84]. A directed-flow-through BES has been tested to maximize the electrode surface area exposed to the electrolyte, achieving productivities comparable to syngas fermentation [85]. However, the culture medium flowing through the path packed with electrodes and biofilm may limit mass transfer between the gaseous and liquid phases, particularly when using low-solubility gas substrates such as CO. An alternative approach to maximize the efficiency of extracellular electron transfer in a BES reactor is to recirculate the headspace gas within the reactor and prevent the loss of H<sub>2</sub> produced at the cathode [86].

The position of the reference electrode is crucial for accurate analysis of a BES [87] (**Figure 10**). The reference electrode should be placed outside the current path, yet as close as possible to the working electrode [87]. Additionally, the ohmic drop, which is based on the distance between the reference electrode and the working electrode, provides good understanding of the electrochemical dynamics in electro-fermentation [87].



**Figure 10. Schematic diagram of the setup for microbial electrosynthesis** including a H-type bioelectrochemical system reactor connected to a potentiostat/galvanostat.

A single-chamber BES reactor without a separator can be employed for electro-fermentation, but it may present challenges in maintaining optimal conditions for specific applications [88, 89]. The system must be continuously sparged to remove oxygen for strict anaerobes [88, 89]. As the cathode potential increases, acetate production may not improve as expected, which is likely due to elevated oxygen evolution at the anode *via* electrolysis [89]. Using a separator in a BES employing anaerobes appears to be indispensable.

A higher electron transfer rate leads to a significant pH increase at the cathode, driven by reduction reactions. Incorporating an ion exchange membrane as a separator offers a more sustainable and efficient solution for managing the pH compared to acid injection, especially for long-term operation of a BES reactor [90]. This approach also allows for optimal utilization of the separator's full capabilities, including preventing oxygen from accessing the cathode chamber and bacterial contamination in the anode chamber.

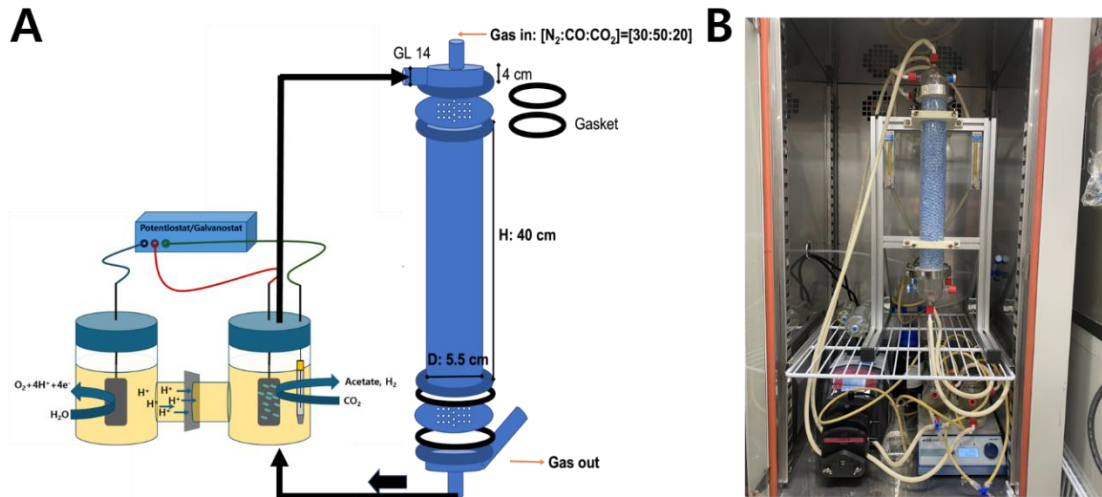
Typically, the separator is positioned on one side of the cathode chamber and connects it to the anode chamber. A difference in local pH can develop on the opposite side of the cathode chamber unless the medium is well-homogenized through stirring or high-speed circulation. To minimize the development of local pH gradients, the cathode can be positioned as close as possible to the proton exchange membrane. The observed increase in pH poses a significant challenge when applying high currents during CO electro-fermentation, as discussed in **Papers I and II**.

While a larger separator surface area enhances proton transfer, the ion exchange membrane may account for more than 10% of a fuel cell's cost [90].

Gas-to-liquid mass transfer is a critical parameter, particularly for gaseous substrates with low solubility such as CO. Trickle bed reactors, commonly used in gas fermentation, rely on a high liquid-to-gas contact area to enhance gas-to-liquid mass transfer. This design facilitates biofilm formation, enabling high cell densities on the material within the catalytic packed bed [36].

In this thesis, a novel CO electro-fermentation reactor setup was developed by integrating a trickle bed reactor with a BES reactor. This configuration aimed to demonstrate CO electro-fermentation while mitigating growth inhibition caused by elevated local pH near the cathode in a BES. The liquid reservoir component of the trickle bed reactor was replaced by an H-type BES reactor, where reductive reactions occurred at the cathode while pH was adjusted through proton supply *via* a proton exchange membrane and possibly with a pH controller. The pH-adjusted medium was then supplied to the top of the catalytic packed bed, ensuring biofilm formation by acetogens in the bed below remaining unaffected. However, the demonstration of this novel CO electro-fermentation system was unsuccessful due to a technical issue: oxygen contamination. (**Figure 11**).

The design of this novel CO electro-fermentation reactor setup was motivated by findings from a previous study, which demonstrated that an exogenous electron mediator was required to observe the positive effects of a BES on CO fermentation [37]. Given that CO is a known inhibitor of hydrogenases [91], I hypothesized the need for an exogenous electron mediator, which might impede extracellular electron uptake. Consequently, I attempted a separation of the CO fermentation part from the bioelectrochemical reaction part (**Figure 10**).



**Figure 11.** Novel CO electro-fermentation reactor set up in our laboratory. (A) Schematic diagram; (B) Photo of the setup.

## 2.2. Host strains and their utilization

Typical bioprocess development procedures include 1) identification of target products and pathways, 2) screening of the potential host strains, and 3) cell factory engineering and process development and engineering. As discussed in **section 2.2.1**, there are several prerequisites for becoming a suitable host strain for CO electro-fermentation. A suitable host strain should be able to utilize electrons originating from electrodes in the fermentation reactor. On the one hand, testing each acetogen for microbial electrosynthesis or CO electro-fermentation would offer direct evidence of which strain performs better in a BES reactor. On the other hand, obtaining clues from bacterial communities capable of CO electro-fermentation, would eliminate the need for the high-throughput screening method necessary when testing single strains. Acetogenic strains and bacterial communities used for MES and CO electro-fermentation are reviewed in **section 2.2.2**, along with a detailed analysis in **Paper I**. Finally, the electro-active acetogen *C. ljungdahlii* will be introduced in **section 2.2.3** as the host strain for the remaining work in thesis.

### 2.2.1. Potential host strains

Since the first demonstration of microbial electrosynthesis, several acetogenic strains have been tested in a BES reactor [28, 62, 92, 93]. *Sporomusa ovata* showed the highest acetate production rate and coulombic efficiency, even with a cathode potential as low as  $-0.9$  V vs Ag/AgCl [62, 93, 94]. *A. woodii* was not initially thought capable of utilizing electrons from the cathode [28]. However, a latter study showed that it could derive them from  $H_2$  produced

on the cathode or use those released by *Desulfopila corrodens* IS4 in the same BES reactor [14, 95]. Microbial electrosynthesis studies using mixed cultures showed *Acetobacterium* spp. were the dominant bacteria [28, 96]. When high salinity conditions were used by a mixed culture to reduce the ohmic resistance of the BES, *Acetobacterium* spp. seemed to be the main for microbial electrosynthesis [77]. This finding looks reasonable considering that *Acetobacterium* species can tolerate alkaline conditions of up to pH 8.5 due to a Na<sup>+</sup>-translocating ATPase, which is not coupled to the proton ion gradient across the cell's plasma membrane [97, 98]. A recent study showed that *E. limosum* was one of the dominant species in a microbial electrosynthesis study using a mixed culture, where it showed a productivity comparable to that of syngas fermentation [85]. *E. limosum*, which has not been tested for microbial electrosynthesis using the pure culture, is a known CO-fermenting acetogen. It also has a Na<sup>+</sup>-dependent energy conservation system and optimal pH of up to 7.2 [27, 99]. Overall, the absence of a H<sup>+</sup>-dependent energy conservation system in some acetogens, which might otherwise be disrupted under elevated pH and low extracellular H<sup>+</sup>, could contribute to their tolerance of high electric currents in a BES reactor. This tolerance may also rely on other factors, such as Na<sup>+</sup>-dependent energy systems and adaptations to BES conditions. Nonetheless, to achieve the same productivity, it is not essential for CO electro-fermentation to have an electric current density as high as that required for CO<sub>2</sub>-based microbial electrosynthesis (see **section 2.1.1**).

Only a few known electro-active acetogens can utilize CO as both a carbon and electron source. CO can inhibit certain enzymatic reactions in bacterial cells, which may halt cell growth. Specifically, because CO is a known competitive inhibitor of hydrogenases [91], it might also inhibit extracellular electron uptake [37]. When CO electro-fermentation was demonstrated for the first time using a mixed culture, no sustainable metabolites were observed without the use of an exogenous electron mediator [37]. The authors suggested that the inhibitory effect of CO could be circumvented by employing an electron mediator [37].

### **2.2.2. *Clostridium autoethanogenum* and *Clostridium ljungdahlii***

*C. autoethanogenum* is a versatile acetogenic bacterium with significant potential for industrial application in biofuel production and carbon capture. It is capable of fermenting CO and/or H<sub>2</sub>:CO<sub>2</sub> using the Wood-Ljungdahl pathway. This organism has garnered attention for its remarkable ability to produce ethanol from CO, along with acetate, lactate, and 2,3-butanediol as natural metabolites [18]. The metabolic engineering of *C. autoethanogenum* has become possible due to several advancements in genetic manipulation, a better understanding of its metabolic pathways, and the development of synthetic biology tools [100-102]. *C. autoethanogenum* was also tested in a BES reactor with several exogenous synthetic electron mediators using fructose as carbon source [103]. *C.*

*autoethanogenum* was unable to utilize electrons supplied by the electrode unless an exogenous electron mediator with a redox potential below that of ferredoxin ( $E_0' = -500 \sim -400$  mV vs standard hydrogen electron, SHE) was used [103]. Another study claimed that *C. autoethanogenum* was an electro-active bacterium capable of performing electro-fermentation without the need for an exogenous electron mediator [104]. However, no studies have yet demonstrated microbial electrosynthesis from  $\text{CO}_2$  using *C. autoethanogenum* [103, 104].

Conversely, electro-active *C. ljungdahlii*, which is genetically similar to *C. autoethanogenum*, has been shown to carry out microbial electrosynthesis [22, 28, 80, 83, 105-107]. The high genetic similarity between these two *Clostridium* species makes it difficult to distinguish them *via* 16S rRNA analysis [106, 107].

In this thesis, *C. ljungdahlii* was selected as the host strain for microbial electrosynthesis due to its industrial relevance, ability to ferment CO and produce ethanol, and its electro-active properties.

### 2.2.3. Bacterial communities used for CO electro-fermentation

The first demonstration of CO electro-fermentation using a mixed culture highlighted the potential for combining gas fermentation with electro-fermentation technology [37]. In that study, anaerobic digestion sludge taken from a wastewater treatment plant was used as the inoculum for CO electro-fermentation, and the resulting bacterial communities developed with or without application of electricity were analysed [37]. The two bacterial communities enabled the growth of different acetogen species, with neutral red acting as an exogenous electron mediator [37]. *Sporomusa* spp. and *Treponema* spp. were found in an open circuit (without electricity) [ $\text{N}_2:\text{CO}:\text{CO}_2$ ]-fed BES reactor [37]. *Sporomusa* spp. are known electro-active acetogens that ferment  $\text{H}_2:\text{CO}_2$  but do not utilize CO [28, 93]. *Treponema* spp. are also  $\text{H}_2:\text{CO}_2$  fermenting acetogens incapable of CO oxidation (Graber, Leadbetter et al. 2004). Therefore, it is highly possible that the bacterial community developed in the open circuit reactor did not contain CO-oxidizing acetogens, but  $\text{CO}_2$ -fixing acetogens. These could have used  $\text{H}_2$  produced by the water-gas shift reaction of *Desulfovibrio* spp. (**Equation 14**) [108].



Sulfate-reducing *Desulfovibrio* spp. were indeed found both with and without electricity [37, 109]. Electro-active *Desulfovibrio* spp. can produce  $\text{H}_2$  at the cathode in a BES reactor [110].

*Alkalibaculum* spp. and *Acetobacterium* spp. were the dominant acetogen species in a [ $\text{N}_2:\text{CO}:\text{CO}_2$ ]-fed BES reactor with electricity plus neutral red [37]. *Alkalibaculum* spp. are

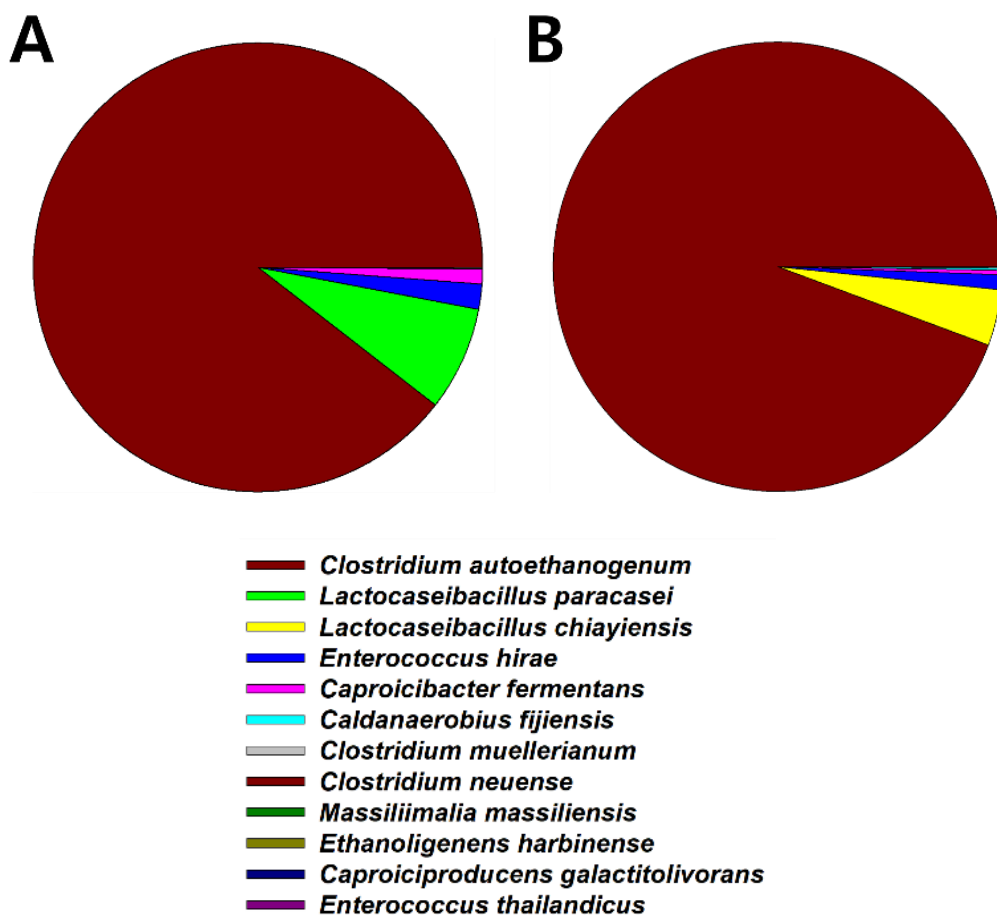
known CO-fermenting acetogens that thrive in alkaline conditions [16]. *Acetobacterium* spp. was also highly dominant in another CO electro-fermentation study using a different ratio of CO:CO<sub>2</sub> mixed gas [68]. *Acetobacterium* spp. can ferment H<sub>2</sub>:CO<sub>2</sub> or H<sub>2</sub>:CO<sub>2</sub> plus CO, but cannot grow on 100% CO without another carbon and electron source [111, 112]. That might explain why coulombic efficiencies observed in the study were below 100% [68]. *Alkalibaculum* spp. and *Acetobacterium* spp. possess the required tolerance towards alkaline pH caused by reductive reactions at the cathode in a BES reactor (**section 2.2.1**). Hence, *Alkalibaculum* spp. Was likely the main CO-oxidizing acetogen in the above study [37]. Nonetheless, neutral red present in the medium can also deliver electrons from the cathode to *Acetobacterium* spp. Therefore, it is difficult to conclude which is the exact mechanism of CO electro-fermentation at such high coulombic efficiency (>190 %) [37].

Several challenges to bacterial cultures in CO electro-fermentation were identified from the start, including reliance on an exogenous electron mediator and production of short-chain fatty acids only [37]. However, the complexity of the bacterial community made identifying the exact mechanism of CO electro-fermentation challenging [37]. Other CO electro-fermentation studies used inoculi taken from anaerobic environments in BES reactors [66-68].

In **Paper I**, pre-enrichment of a mixed culture under CO conditions diminished the complexity of the bacterial community and helped elucidate the mechanism of CO electro-fermentation (**Paper I**). Metagenomic 16S rRNA analysis showed that the bacterial community pre-enriched under CO conditions was dominated by *C. autoethanogenum*, which accounted for more than 89% of it (**Figure 11**). When the pre-enriched bacterial community was tested for both microbial electrosynthesis and CO electro-fermentation, it utilized the electrons from the electrode even in the presence of CO, without an exogenous electron mediator (**Paper I**). Furthermore, the pre-enriched bacterial community was able to produce ethanol in a [N<sub>2</sub>:CO:CO<sub>2</sub>]-fed BES reactor at 10 mA (**Figure 8 and 12**).

Given the high genetic similarity and the difficulty in distinguishing between *C. autoethanogenum* and *C. ljungdahlii*, this result confirmed that selecting *C. ljungdahlii*, with its known electro-activity, as the host strain for this thesis work on developing the CO electro-fermentation process was appropriate (**Papers I and II**).





**Figure 12.** 16S rRNA bacterial community analysis results using two different primer sets. (A) V1-V2 (27F-Eub338 primer set); (B) V3-V4 (Bakt\_341F-805R primer set). Data originated from **Paper I**.



## Chapter 3. Extracellular electron transfer

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This chapter describes the mechanisms of extracellular electron transfer and uptake. In this thesis, extracellular electron transfer refers to the process by which microorganisms export electrons from their internal metabolic processes to an external electron acceptor, such as an electrode or a mineral surface. Conversely, extracellular electron uptake refers to the process by which microorganisms accept electrons from an external electron donor, such as an electrode, to fuel their metabolic activities.

First, well-known extracellular electron transfer pathways employed by typical electro-active bacteria will be discussed. They will be followed then by putative extracellular electron uptake mechanisms in, along with strategies for enhanced electron uptake via these pathways. Finally, I will propose potential approaches for improving electron uptake in acetogens.

### 3.1. Extracellular electron transfer mechanisms

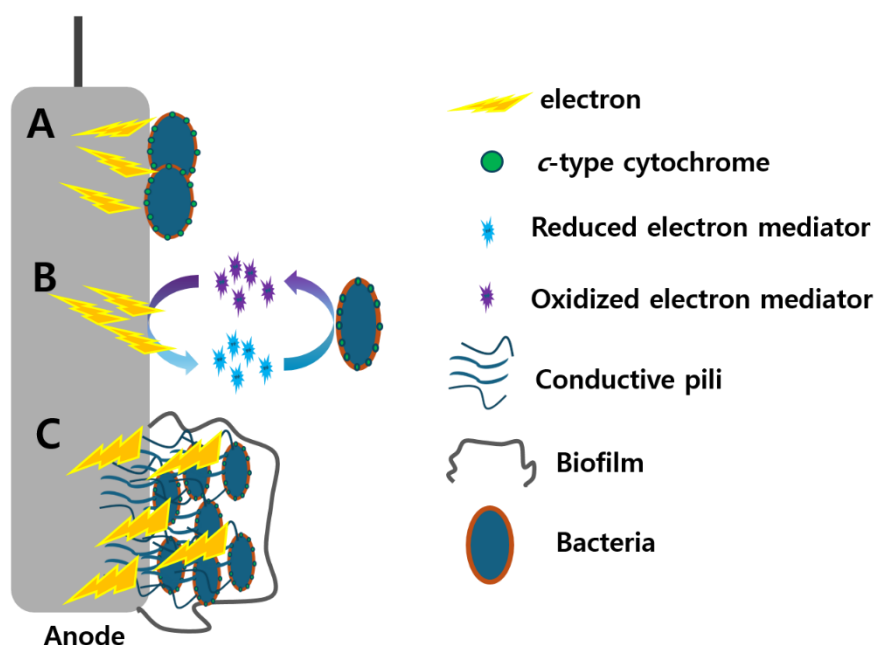
#### 3.1.1. Known mechanisms of type strains

Extracellular electron transfer allows microorganisms to transfer electrons to external solid materials. This is crucial for MFCs and biogeochemical cycles. Extracellular electron transfer mechanisms have been extensively studied in model organisms, such as *G. sulfurreducens* PCA and *S. oneidensis* MR-1 [39, 113]. Species within the genera *Geobacter* and *Shewanella* can perform anaerobic respiration by linking their metabolic processes to abiotic, insoluble, solid-state electron acceptors (e.g., metal oxides and electrodes in a BES) using *c*-type cytochromes [39]. *C*-type cytochromes play a key role in extracellular electron transfer due to their substrate diversity and localization within the periplasmic compartment and on the cell surface [114]. Surface-localized *c*-type cytochromes interact directly with extracellular electron acceptors, such as metal oxides, electrodes, or other solid-phase electron sinks. Meanwhile, periplasmic *c*-type cytochromes facilitate electron transfer between the inner and outer membranes. Also, *c*-type cytochromes are defined by the covalent attachment of heme groups to their polypeptide chains via thioester bonds in a conserved CXXCH motif [115]. This structural feature allows them to interact with a wide range of substrates by facilitating electron transfer or redox reactions,

enabling such organisms to survive in environments with varying electron acceptors or donors [116].

There are three primary extracellular electron transfer mechanisms utilized by electro-active bacteria: 1) direct electron transfer via outer membrane cytochromes, 2) electron transfer mediated by electron shuttles, and 3) long-range electron transfer through a conductive biofilm matrix made of cytochrome nanowires [117]. *Geobacter* species utilize primarily direct electron transfer and conductive nanowires, which are made possible by the large number of *c*-type cytochromes encoded in their genome [118]. Unlike some anaerobic bacteria, which rely solely on fermentation, an abundance of *c*-type cytochromes allows *G. sulfurreducens* to oxidize acetate and other electron donors *via* a modified TCA cycle. Accordingly, acetate assimilation depends on a CoA transferase and the regeneration of intracellular electron carriers such as  $\text{NAD}^+$  by extracellular electron transfer [119] (**Figure 13**).

*S. oneidensis* MR-1 exploits a wide range of substrates and flexible extracellular electron transfer mechanisms. Specifically, it naturally secretes flavins as electron shuttles, enabling efficient electron transfer to poorly soluble electron acceptors such as Fe(III) oxide [120]. The secreted flavins are integrated within *Shewanella*'s biofilm matrix, further facilitating electron transfer [121]. This versatile extracellular electron transfer mechanism allows *S. oneidensis* to thrive in diverse habitats such as interfacial environments, where conditions can rapidly shift between oxic and anoxic [120].



**Figure 13. Schematic diagram of extracellular electron transfer mechanisms of type electro-active bacteria to the anode.** (A) Direct electron transfer, (B) mediated electron transfer, and (C) long-range electron transfer.

Extracellular electron transfer mechanisms of electro-active bacteria have been studied using various experimental techniques and approaches. These studies are often conducted in a BES, which provides controlled conditions and simplifies electrochemical analysis. Cyclic voltammetry is one of the most widely used electrochemical techniques, providing valuable information about redox processes and electron transfer kinetics [70]. For example, cyclic voltammetry can be used to identify the key electron donor and acceptor in a reaction by comparing substrate turnover and non-turnover conditions [122, 123]. It also helps determine the involvement of electro-active biofilms in bioelectrochemical reactions and at which potential these occur [123], as well as whether the reaction is mass-transfer-limited or not [70]. Another commonly used method is electrochemical impedance spectroscopy. The measurement of biofilm capacitance indicates biofilm thickness or mass, as well as charge transfer and diffusion resistance [122, 124]. Molecular biology approaches such as gene deletion have also been employed to better understand extracellular electron transfer mechanisms [125]. Deletion of the GSU1771 gene in *G. sulfurreducens* improved biofilm formation and transcription of genes involved in extracellular electron transfer (e.g., *c*-type cytochromes and *pilA*) and increased electrochemical response during cyclic voltammetry analysis [126]. ALE can be another powerful tool for identifying extracellular electron transfer mechanisms, particularly when the genetic basis of the desired trait is not fully understood [127, 128] (**Paper III**).

### 3.1.2. Engineering extracellular electron transfer

Engineering extracellular electron transfer mechanisms improves the efficiency, versatility, and scalability of BESs [129]. Optimized extracellular electron transfer can lead to faster removal of organic pollutants or toxic metals in wastewater treatment plants, improved current generation for biosensors or MFCs, and better interactions between electro-active bacteria for electro-fermentation [130].

Electro-active bacteria can be engineered to enhance or elucidate specific extracellular electron transfer pathways, improving our understanding of the underlying mechanisms. The availability of advanced genetic engineering tools provides opportunities to identify and optimize key genes and proteins involved in electron transfer. *Shewanella* species are genetically more tractable than *Geobacter* ones because they benefit from a wider availability of genetic tools such as plasmid-based systems, are more amenable to transformation and gene manipulation, and are easier to cultivate under aerobic conditions [131]. As a result, many scientists studying extracellular electron transfer mechanisms prefer *Shewanella* species for their work [131]. This has resulted in extensive knock-out collections [132]. Additionally, both homologous and heterologous overexpression studies have been attempted to enhance electricity generation. For example, homologous overexpression of

cymA, a tetraheme *c*-type cytochrome anchored to the inner membrane of *S. oneidensis* MR-1, led to higher power density and faster growth [46]. Furthermore, *S. oneidensis* MR-1 was engineered to co-express its own citrate synthase and succinyl-CoA:acetate CoA-transferase from *G. sulfurreducens* [47]. The engineered strain generated higher power density than the wild type, even when lactate was used as a carbon source [47]. Extracellular electron transfer pathways can be expressed in a domesticated bacterial chassis to facilitate their cultivation and genetic manipulation. *Pseudomonas putida* KT2440, a strict aerobe, was engineered to produce phenazine as an electron mediator, enabling growth under oxygen-limited conditions [133]. *Escherichia coli* has been engineered to express conductive protein nanowires, whose physical properties were comparable to those of *G. sulfurreducens* nanowires [134].

Extracellular electron transfer can be enhanced through both biofilm matrix engineering and electrode surface modification [135]. Given that the biofilm of *S. oneidensis* MR-1 is a key element of extracellular electron transfer, one strategy for improving conductivity and therefore electron transfer is to coat the cells with polypyrrole. This can be achieved via *in situ* polymerization, using Fe<sup>3+</sup> functionalized bacterial cells as oxidative initiators for pyrrole polymerization [136, 137]. Another approach to boost electricity generation is to immobilize cells on electrode surfaces [129]. Electrode modification for type electro-active bacteria in BESs includes the introduction of functional groups on the electrode surface to enhance material wettability, thereby improving bacterial adhesion and extracellular electron transfer efficiency [138].

## 3.2. Extracellular electron uptake by acetogens

While known electro-active bacteria, such as *S. oneidensis* and *G. sulfurreducens*, carry out mostly extracellular electron transfer shuttling electrons from intracellular metabolic pathways to external electrodes by oxidizing organic substrates—certain autotrophic microorganisms rely on perform extracellular electron uptake, whereby electrons flow *into* the cell from an external electrode. Extracellular electron uptake supports autotrophic metabolism by reducing CO<sub>2</sub>, while also synthesizing value-added chemicals. Acetogens have attracted particular attention for their ability to fix CO<sub>2</sub> and produce acetate or ethanol using electrons supplied by an electrode connected to an external power supply [38]. This process holds both industrial and ecological relevance, offering a potential route for carbon-neutral bioprocessing and sheds light on how organisms adapt to use external electrons in natural environments [139]. Unlike the well-characterized extracellular electron transfer mechanisms of electro-active bacteria, the identification of unknown electron transfer components involved in extracellular electron uptake in autotrophic bacteria remains a challenge due to limited genetic engineering tools and difficult cultivation [139]. **Section**

**3.2.1** revisits what is known about extracellular electron uptake in autotrophs; whereas **section 3.2.2** discusses how to identify and improve EEU using *C. ljungdahlii*.

### 3.2.1. Known aspects of electron uptake and hypotheses

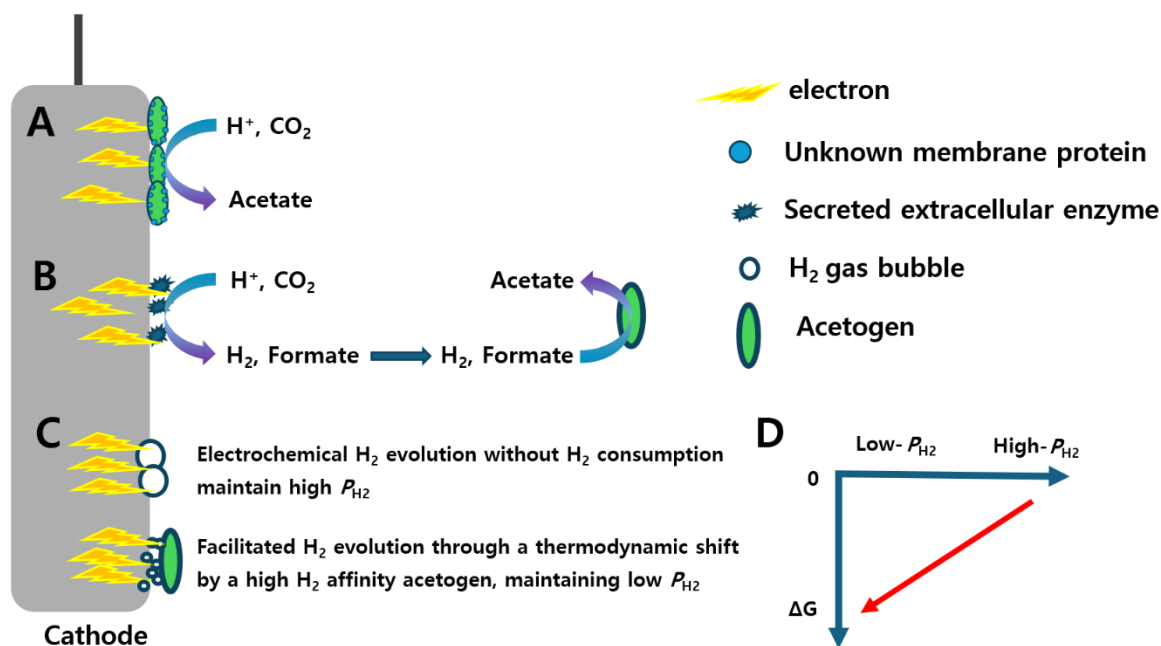
When microbial electrosynthesis using an acetogen was first demonstrated, the working electrode potential was set to  $-400$  mV vs SHE [62]. This was done to avoid  $H_2$  evolution, which had been empirically observed at  $-600$  mV vs SHE [140]. Given that the biological standard potential for  $H_2$  evolution is  $-414$  mV vs SHE (**Equation 10**), setting the cathode to  $-400$  mV vs SHE helped maintain high coulombic efficiency by favoring direct electron transfer and minimizing  $H_2$  formation [62, 141]. Even though no direct electron uptake mechanism by acetogens has been discovered yet, several hypotheses have been suggested to explain how acetogens utilize electrode-derived electrons.

Secretion of electro-active extracellular enzymes is a suggested extracellular electron uptake mechanism [142]. Spent cell-free culture media from *Methanococcus maripaludis* and *Sporomusa sphaeroides* were tested in a BES, revealing significant  $H_2$  and formate production [142]. These bacteria were hypothesized to use the  $H_2$  and formate, generated by secreted electro-active extracellular enzymes, as electron intermediates [142]. Although the spent medium exhibited heat and proteinase sensitivity, no specific proteins were identified [142]. A subsequent study on the spent medium of *S. ovata* suggested that the secreted extracellular enzymes originated during cell lysis [94].

Another interesting hypothesis posits that a thermodynamic shift caused by  $H_2$  consumption on the part of acetogens boosts current consumption in a BES [143]. The onset potential of  $H_2$  evolution ( $E_{H^+/H_2}^\circ$ ) is  $-0.414$  V vs SHE under biological standard condition (pH 7.0, 1 atm  $H_2$ ). The onset potential of  $H_2$  evolution ( $E_{H^+/H_2}$ ) in a BES can be adjusted using Nernst equation, with ideal gas constant  $R$  ( $8.314 \times 10^{-3}$  kJ/mol/K), temperature  $T$  (K), Faraday constant  $F$  (96.485 kJ/V),  $H_2$  partial pressure  $p_{H_2}$  (atm), and proton concentration  $[H^+]$  (M):

$$E_{H^+/H_2} = E_{H^+/H_2}^\circ - \frac{RT}{2F} \ln\left(\frac{p_{H_2}}{[H^+]^2}\right) \quad \text{(Equation 15)}$$

As a result, the onset potential of  $H_2$  evolution becomes  $-0.149$  V vs SHE when the  $H_2$  partial pressure is as low as  $10^{-5}$  atm. Jo Philips suggested that  $H_2$  consumption by acetogens on the cathode sustained  $H_2$  evolution and discrepancies in performance among acetogens depended on their specific  $H_2$  consumption [143] (**Figure 14**). Recently, the  $H_2$  consumption thresholds of different acetogens were characterized [144]; however, the comparative performances of these bacteria in a BES have not yet been systematically evaluated [144, 145].



**Figure 14. Schematic diagram of hypothesized mechanisms for extracellular electron uptake by acetogens from the cathode.** (A) Direct electron uptake from the cathode. (B) Secreted extracellular enzyme-mediated electron uptake. (C) Facilitated  $H_2$  evolution through a thermodynamic shift by a high- $H_2$  affinity acetogen. (D) Shift in the Gibbs free energy of  $H_2$  evolution as a function of  $H_2$  partial pressure.

An engineered *C. ljungdahlii* heterologously expressing formate dehydrogenase demonstrated increased electricity generation in a MFC [146]. In spite of presenting lower  $H_2$  affinity compared to *A. woodii*, *C. ljungdahlii* exhibited electroactivity in microbial electrosynthesis, which *A. woodii* did not [28]. This points to a possibly unknown extracellular electron uptake mechanism in *C. ljungdahlii* beyond  $H_2$ -mediated electron transfer. Conversely, *C. autoethanogenum* can utilize electrons from the electrode only when a synthetic electron mediator with a redox potential lower than that of ferredoxin is used [103].

Zerovalent iron has been used to isolate electro-active acetogens, because it can act as a solid-state electron donor in aqueous solution, similar to the cathode in a BES [69, 147]. Bacterial communities enriched from microbial electrosynthesis are highly similar to those enriched using zerovalent iron as a solid-state electron donor in serum bottles [96, 141, 147, 148], suggesting a common extracellular electron uptake mechanism for cathode oxidation in a BES and iron oxidation in serum flasks.

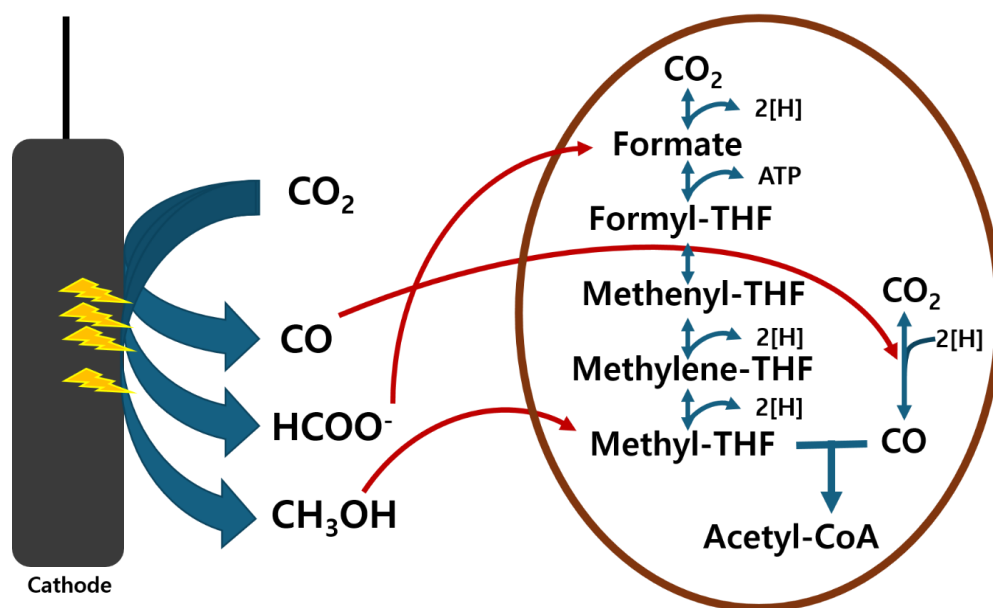


### 3.2.2. Means to improve electron uptake by electrochemical approaches

A BES utilizes electro-active bacteria as biocatalysts. Consequently, extracellular electron uptake can be improved *via* two main routes: electrochemical system engineering or microbial cell factory engineering. The first focuses on optimizing electrode materials and designs to enhance electrochemical reactions and attempts to employ electrodes for CO<sub>2</sub> conversion to value-added chemicals have been made.

Highly porous electrodes provide increased surface area for biofilm development, which is advantageous for microbial electrosynthesis using mixed cultures [149]. Additionally, carbon electrodes can be coated with metal catalysts to facilitate electrochemical reactions that produce electron intermediates. For example, a carbon felt electrode was modified by electrodepositing nickel phosphide to enhance H<sub>2</sub> evolution on the cathode [83]. Another study demonstrated the use of a carbon felt coated with Cobalt (II) phthalocyanine catalyst as the electrode for *in situ* production of syngas, which was subsequently used for microbial electrosynthesis with *C. ljungdahlii* [105]. The electrochemical conversion of CO<sub>2</sub> to soluble electron-carrying intermediates might improve performance of microbial electrosynthesis. Formate is a common CO<sub>2</sub>-derived product during microbial electrosynthesis using CO<sub>2</sub>, provided an appropriate electrode is employed. As a proof of concept, modified electrodes were tested for formate production from CO<sub>2</sub> for acetate and polyhydroxybutyrate production [150-152]. Similarly, electrochemical conversion of CO<sub>2</sub> to methanol represents an alternative strategy to enhance extracellular electron uptake. Several acetogens, such as *A. woodii*, *E. limosum*, and *S. ovata*, can assimilate methanol into the Wood-Ljungdahl pathway [153].

Methanol assimilation for acetate production provides a higher net ATP yield (0.83–0.92 mol ATP/mol acetate) compared to acetate production from H<sub>2</sub> and CO<sub>2</sub> (0.75 mol ATP/mol acetate) [153, 154]. Consequently, electrochemically produced methanol could improve cell growth and coulombic efficiency in microbial electrosynthesis. Given that soluble electron-carrying intermediates such as methanol are less volatile than gaseous electron mediators, their use could increase the efficiency of microbial electrosynthesis compared to CO<sub>2</sub>-based processes (**Figure 15**).

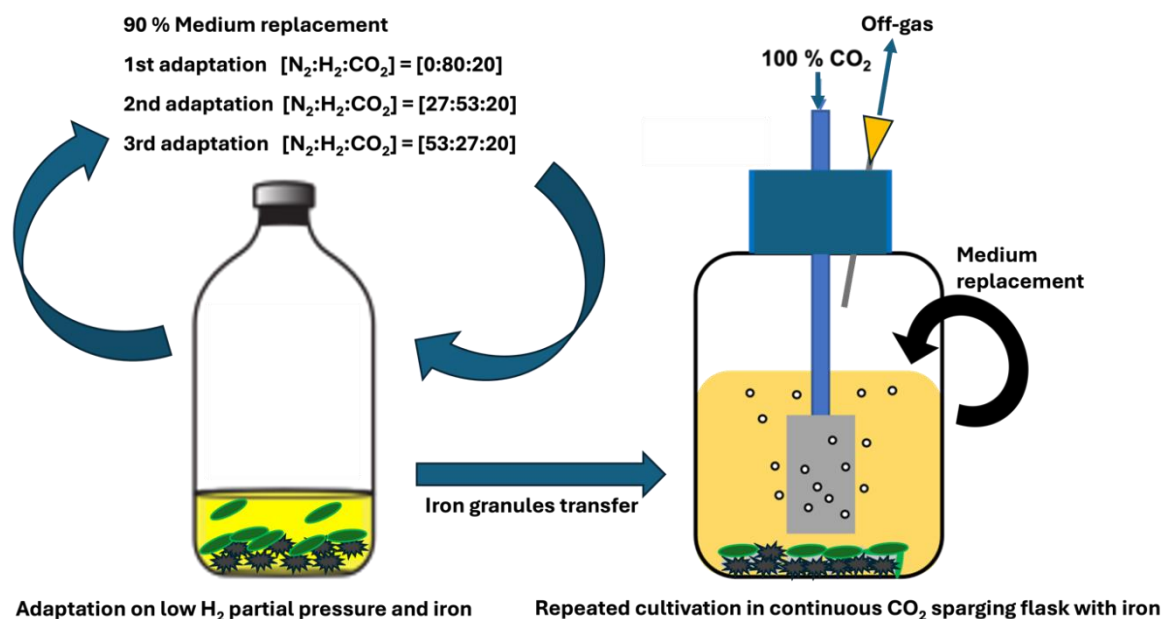


**Figure 15. Schematic diagram of CO<sub>2</sub> reduction to CO, formate, and methanol by electrochemical reactions and their assimilation into the Wood-Ljungdahl pathway.**

Microbial cell factory engineering to improve extracellular electron uptake by acetogens will become feasible once the underlying extracellular electron uptake mechanisms are better understood. One potential approach is the use of a transposon system to create a gene knock-out collection. A transposon system was employed to identify essential genes for autotrophic growth in *C. autoethanogenum* [155]. This method could similarly be applied to uncover unknown extracellular electron uptake mechanisms in acetogens. However, effective implementation of this approach would require a high-throughput screening system. Several high-throughput platforms have been tested in BES studies [156, 157], including for a microbial electrolysis cell without a separator [158]. However, this solution may not be suitable for microbial electrosynthesis by strict anaerobic acetogens due to oxygen evolution at the anodes.

### 3.2.3. Adaptive laboratory evolution to improve electron uptake

Adaptive laboratory evolution (ALE) is an experimental technique used to study the evolutionary processes of microorganisms and improve their traits for specific applications. It involves subjecting microbial populations to controlled environmental conditions over extended periods, allowing them to adapt and evolve desired characteristics through natural selection. ALE can also be employed to identify unknown extracellular electron transfer mechanisms and to enhance the performance in a BES (**Paper III**). When *G. sulfurreducens* was cultivated in a long-term in a MFC and in a serum flask with Fe(II) oxide, electricity generation by the evolved strains both in a MFC and in a serum flask with Fe(II) oxide significantly improved [127, 128]. The studies found that different expression levels of

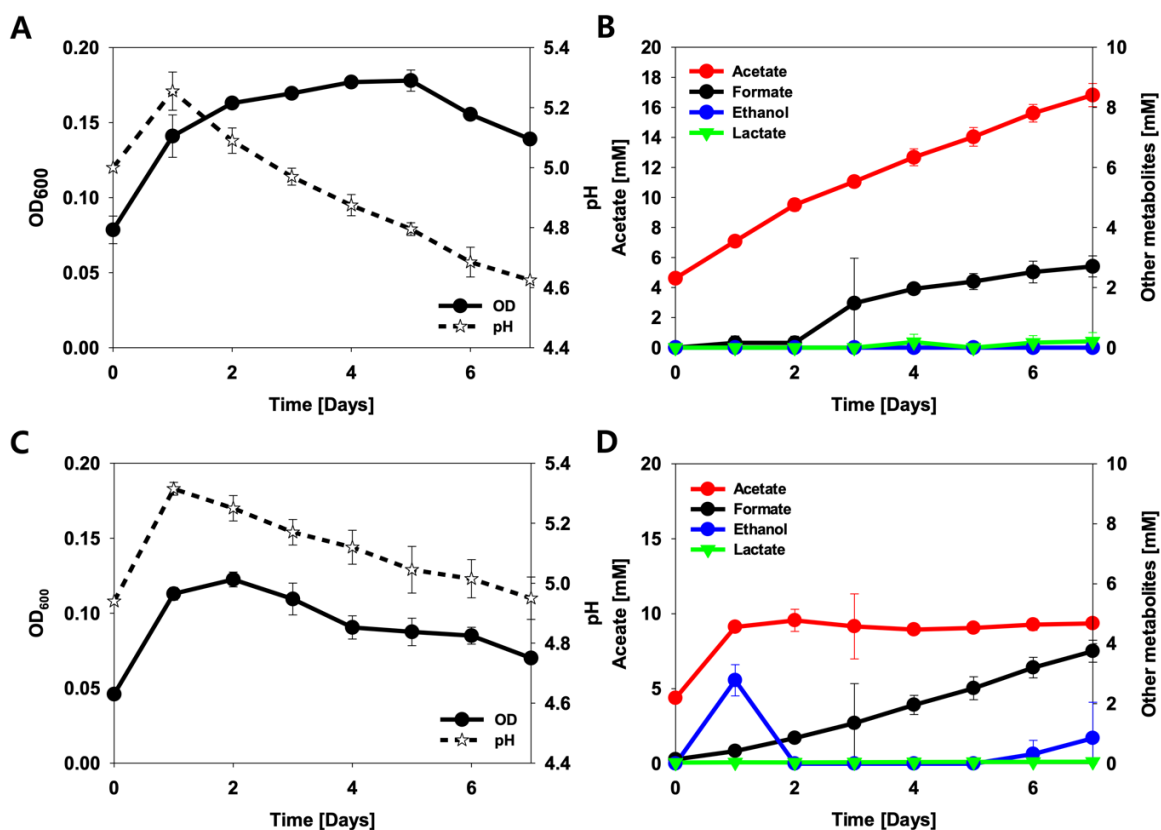


**Figure 16.** Schematic diagram of the setup used for evolutionary adaptation of *C. ljungdahlii* on iron in a continuously CO<sub>2</sub>-sparged flask.

*c*-type cytochromes of the evolved *G. sulfurreducens* strains after ALE [127, 128]. Also, ALE of *S. ovata* under methanol condition accelerated its autotrophic metabolism using the Wood-Ljungdahl pathway, leading to the improved performance in a BES condition [159].

In **Paper III**, *C. ljungdahlii* incubated in medium containing iron granules was initially adapted to decreasing amounts of H<sub>2</sub> in the headspace. The iron granules with attached *C. ljungdahlii* cells were subsequently transferred to a continuously CO<sub>2</sub>-sparged flask for further adaptation and cultivation. *C. ljungdahlii* was repeatedly cultivated in the same flask with medium replacement to facilitate evolutionary adaptation (**Figure 16**).

After cultivation was repeated for the 38th time, acetate production by the adapted culture was higher compared to that in the first cultivation (**Figure 3 in Paper III**). Isolated mutants were tested under different conditions, including fructose, H<sub>2</sub>:CO<sub>2</sub>, and CO<sub>2</sub> with iron. Surprisingly, all isolated mutants performed worse than the wild-type under H<sub>2</sub>:CO<sub>2</sub> and CO<sub>2</sub> with iron (**Paper III**). Whole-genome sequencing revealed that the mutations were likely an adaptive response to harsh electron-limited conditions, allowing cells to save resources, such as amino acids and ATP (Table from the manuscript). One mutant was tested in a BES but exhibited poor growth, formate and ethanol accumulation, and early cessation of acetate production (**Figure 17**). *Rhodobacter spheroides* is known to accumulate polyhydroxybutyrate under nutrient limitation [160] or in a BES, compared to typical cultivation conditions [161]. Drawing on this analogy, it is possible that *C. ljungdahlii* may have evolved to store electrons from volatile electron donors such as H<sub>2</sub> in the form of



**Figure 17. Growth (OD), medium pH, and metabolite profiles of *C. ljungdahlii* in BES reactors. (A, B) Wild-type, (C, D) isolate #8. Error bars denote standard deviation (n = 2). Figure taken from **Paper III**.**

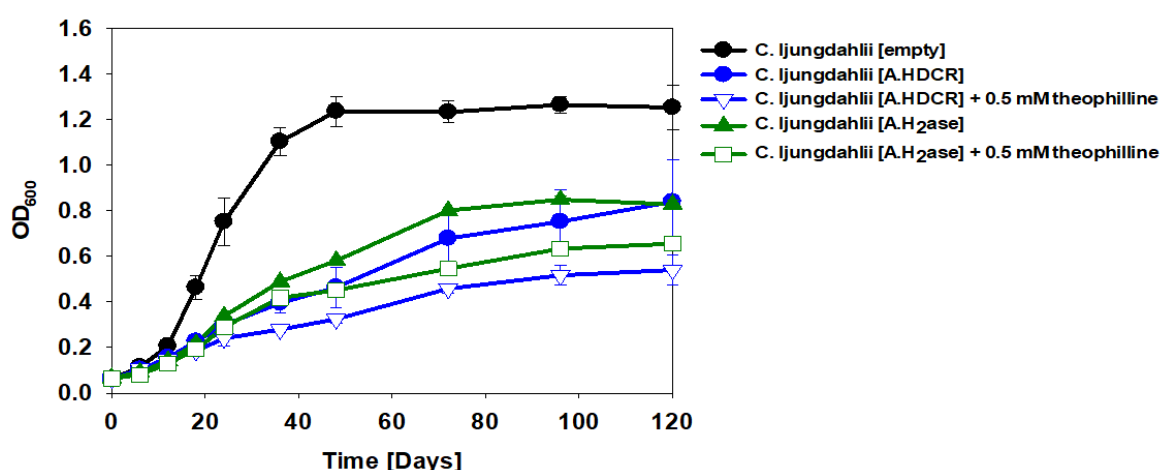
soluble chemicals, which could be metabolized under more favorable conditions in the future (**Figure 17**).

Although the experimental results did not meet expectations, alternative approaches to evolutionary adaptation can now be proposed based on the insights gained from **Paper III**. One suggestion is to include yeast extract in the medium along with iron. In **Paper III**, yeast extract was omitted to encourage *C. ljungdahlii* to rely solely on iron as the electron donor, with the aim of maximizing iron utilization. However, whole-genome sequencing revealed that the poor nutritional conditions likely hindered the desired mutations (**Table 3** in **Paper III**). An alternative strategy would involve cultivating *C. ljungdahlii* under continuous operation, with optimized operational parameters to have better cell growth (**section 2.1.1**), in a BES or in a bioreactor with a gradually decreasing partial pressure of H<sub>2</sub> to drive adaptive laboratory evolution. The latter is based on the assumption that extracellular electron uptake is mediated solely by H<sub>2</sub>. These approaches aim to overcome the challenges observed in **Paper III** and ameliorate evolutionary adaptation.

### 3.2.4. Means to improve electron uptake by biotechnological approaches

Rational engineering approaches can also be employed. The utilization of electrochemically produced formate could be enhanced if the biocatalyst for microbial electrosynthesis is metabolically engineered. Formate plays a crucial role as an intermediate in bacterial metabolism, making it a promising substrate for engineered pathways to improve efficiency and product yields in microbial electrosynthesis [31, 152]. For instance, formate can be utilized by formate dehydrogenase engineered to have different specificity for  $\text{NAD}^+$  or  $\text{NADP}^+$  and thus supply appropriate intracellular electron carriers [162]. Additionally, formate can be used to enhance gluconeogenesis by pyruvate formate lyase instead of pyruvate ferredoxin oxidoreductase, which uses reduced ferredoxin for pyruvate synthesis (**Paper IV**).

Heterologous expression of high-affinity hydrogenase can be attempted to improve EEU in *C. ljungdahlii*. The high affinity for  $\text{H}_2$  of some acetogen strains is assumed to come from high- $\text{H}_2$  affinity hydrogenase. One of the key findings from **Paper II** was the role of  $\text{H}_2$  in mediating EEU by *C. ljungdahlii*. Consequently, the heterologous expression of a hydrogenase with even greater  $\text{H}_2$  affinity could potentially enhance EEU by *C. ljungdahlii*. For this thesis work, potential candidates with such characteristics were chosen among hydrogenases from *A. woodii* strain with a significantly lower  $\text{H}_2$ -threshold than *C. ljungdahlii* using HydDB [144, 163]. However, cloning was challenging in some cases as it involved lengthy attempts to regulate multi-subunit hydrogenase operons. Improper modulation of multi-subunit hydrogenases resulted in significantly slower cell growth compared to the control (**Figure 18**).



**Figure 18.** OD measurement of *C. ljungdahlii* [empty] and *C. ljungdahlii* [A.HDCR] under  $\text{H}_2:\text{CO}_2$  conditions with or without 0.5 mM theophylline as inducer. *C. ljungdahlii* [empty] carried the pMTL83121-Pfdx-E\_empty plasmid; whereas *C. ljungdahlii* [A.HDCR] carried the pMTL83121-Pfdx-E\_A.HDCR plasmid.



## Chapter 4. Metabolic engineering for electro-fermentation

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All acetogens belong to the phylum Firmicutes, with most of them falling in the class Clostridia [35]. Notably, many medically and industrially relevant strains are members of the genus *Clostridium* [164], which has led to the development of specific biotechnology tools and the genetic engineering of some acetogens [164, 165]. Nevertheless, the number of genetic manipulation studies on acetogens remains scarce. In particular, the limited availability of regulatory elements poses a significant challenge for the expression of certain genes, including pyruvate formate lyase (**Paper IV**).

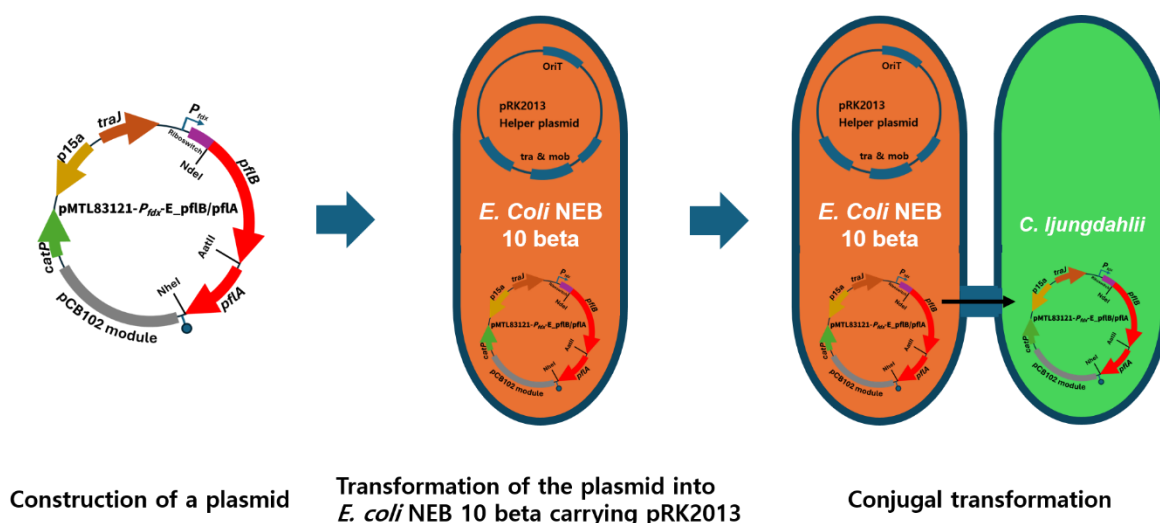
Both microbial electrosynthesis and CO electro-fermentation exhibit lower biomass and slower growth rates in acetogens compared to conventional gas fermentation, which is itself hindered by low biomass and slow growth (**Papers I, II, III, and IV**). This chapter focuses on metabolic engineering approaches aimed at improving electro-fermentation processes. It begins with a review of transformation methods, gene expression regulatory elements, and genome editing tools available for engineering acetogens. Subsequently, strategies to enhance substrate utilization and improve cell growth are explored, including the heterologous expression of pyruvate formate lyase in *C. ljungdahlii* to address the low biomass in gas fermentation and microbial electrosynthesis (**Paper IV**).

### 4.1. Genetic engineering of acetogens

A critical step in advancing the genetic manipulation of acetogens is the development of robust plasmid DNA transfer protocols, as not all acetogens are amenable to plasmid transfer [166]. Many acetogens lack the innate ability to take up extracellular DNA. One exception is *T. kivui*, which can take up DNA during exponential growth [167, 168]. In contrast, naturally competent bacteria such as *Bacillus subtilis* and *Streptococcus pneumoniae* are equipped with specialized systems for DNA uptake, including Com proteins [169, 170]. Additionally, Gram-positive acetogens have a thick peptidoglycan layer that forms a rigid cell wall, requiring specific techniques, such as electroporation with cell wall-weakening agents, to facilitate DNA entry [171]. Acetogens often possess also robust restriction-modification systems that protect them against foreign DNA, rendering transformation a

challenge [168, 172]. These restriction-modification systems can be bypassed by methylating the DNA or using restriction enzyme inhibitors [173, 174]. Plasmid DNA transfer into acetogens can be achieved through electroporation or conjugation (Bourgade, Minton et al. 2021). Electroporation requires a specific device and the preparation of competent cells, which may remain in such state only for a short period [175]. In contrast, conjugal transformation requires a donor strain carrying the plasmid of interest, often accompanied by a helper plasmid to facilitate DNA transfer. However, conjugal transformation includes an additional step to verify that the transformed plasmids are not mutated, as mutations can occasionally occur during the conjugation process [176]. In conclusion, many methods are available, but their application must be validated for every new modification, complicating the genetic transformation of acetogens.

In **Paper IV**, *C. ljungdahlii* was subjected to conjugal transformation with a modified protocol adapted for this and other *Clostridium* species [174, 177]. The helper plasmid pRK2013 was transformed into *E. coli* NEB 10 beta, a strain equipped with DNA adenine and cytosine methylases. Target plasmids were then introduced into *E. coli* NEB 10 beta carrying pRK2013, creating donor strains ready for conjugation. Transfer of target plasmids into *C. ljungdahlii* was achieved using a conjugation protocol originally developed for *C. autoethanogenum*, with appropriate antibiotic selection [174]. To confirm successful plasmid transformation, plasmids extracted from *C. ljungdahlii* transformants were validated by Sanger sequencing. This streamlined method integrated plasmid DNA methylation and donor strain preparation into a single step, eliminating the need for additional devices for plasmid transformation (**Figure 19**).



**Figure 19. Schematic diagram of the conjugal transformation method used in Paper IV.**



Gene expression regulatory elements are indispensable for acetogen engineering because they enable fine-tuning of genetic and metabolic processes. This control is critical for overcoming the inherent limitations of acetogens, such as low biomass yield, slow growth, and poor metabolic flexibility due to low ATP yield during gas fermentation, while facilitating efficient production of target compounds in industrial applications.

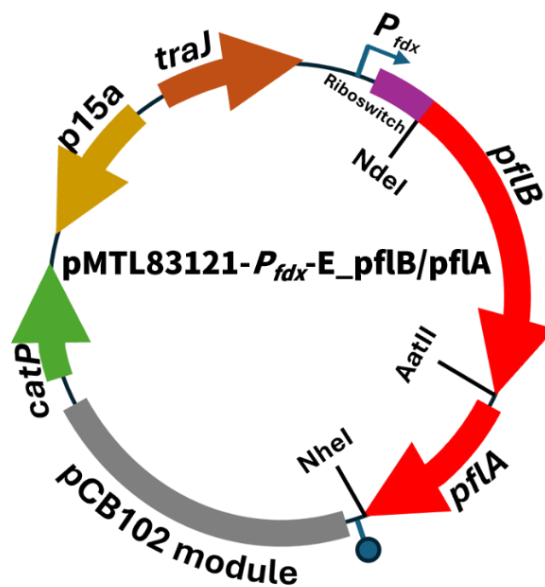
Strong constitutive promoters, such as  $P_{fdx}$ ,  $P_{thl}$ , and  $P_{pta}$ , are widely used in *Clostridium* species to drive gene expression [166, 178]. However, the resulting high levels of gene expression can impose a metabolic burden, leading to ATP limitations, competition for amino acids, and even slower cell growth, particularly in acetogens grown under autotrophic conditions. Constitutive promoters with varying strengths have been developed for *C. ljungdahlii* and *Clostridium acetobutylicum*. However, determining the appropriate expression level often requires labor-intensive testing of constitutive promoters with different strengths and handling of multiple transformants carrying plasmids with these promoters [179]. Inducible promoter systems offer a solution to this challenge by bypassing the need to clone and test plasmids with varying promoter strengths. Several inducible promoter systems have been tested in *C. ljungdahlii*, but none have demonstrated tight regulation in the absence of induction [180, 181]. A tightly regulated expression system is essential when cloning and expressing toxic genes (e.g., for the Cas9 protein), whose constitutive expression may harm the host cell [182, 183].

Cloning and expressing a toxic gene can be achieved also via other strategies. Using a low-copy vector minimizes toxicity by reducing the number of gene templates within the cell [182]. The efficiency of cloning a toxic gene can be further improved by optimizing process conditions, such as adjusting the cultivation temperature or selecting appropriate cultivation media to reduce stress on the cloned strain after transformation [184].

In **Paper IV**, heterologous expression of pyruvate formate lyase was tested in *C. ljungdahlii*. Cloning pyruvate formate lyase proved challenging due to mutations in the start codon sequence (ATG) of the gene in the cloning vector, suggesting potential toxicity associated with its expression (**Figure 20**). Successful cloning was achieved by combining a low-copy number origin of replication (p15a), a theophylline-inducible promoter, and cultivating the transformants at 30 °C (**Figure 21**). Interestingly, background expression of the theophylline-inducible promoter was sufficient to observe the beneficial effects of pyruvate formate lyase on cell growth.



**Figure 20.** The Sanger sequencing result showing the mutation in the start codon (ATG) after cloning of *pflB1* into pMTL83151-*P<sub>thl</sub>*. pMTL83151-*P<sub>thl</sub>* contains ColE1, a high-copy-number origin of replication for gram-negative bacteria, and *P<sub>thl</sub>*, a strong constitutive promoter originated from *Clostridium acetobutylicum*.



**Figure 21.** Plasmid map of pMTL83121-*P<sub>fdx</sub>*-E-*pflB/pflA* used in paper IV to heterologously express pyruvate formate lyase from *A. woodii* in *C. ljungdahlii*. pMTL83121-*P<sub>fdx</sub>*-E contains *p15a*, a low-copy-number origin of replication, and *P<sub>fdx</sub>*-E, a strong constitutive promoter originated from *Clostridium sporogens* under control of a theophylline responsive riboswitch.

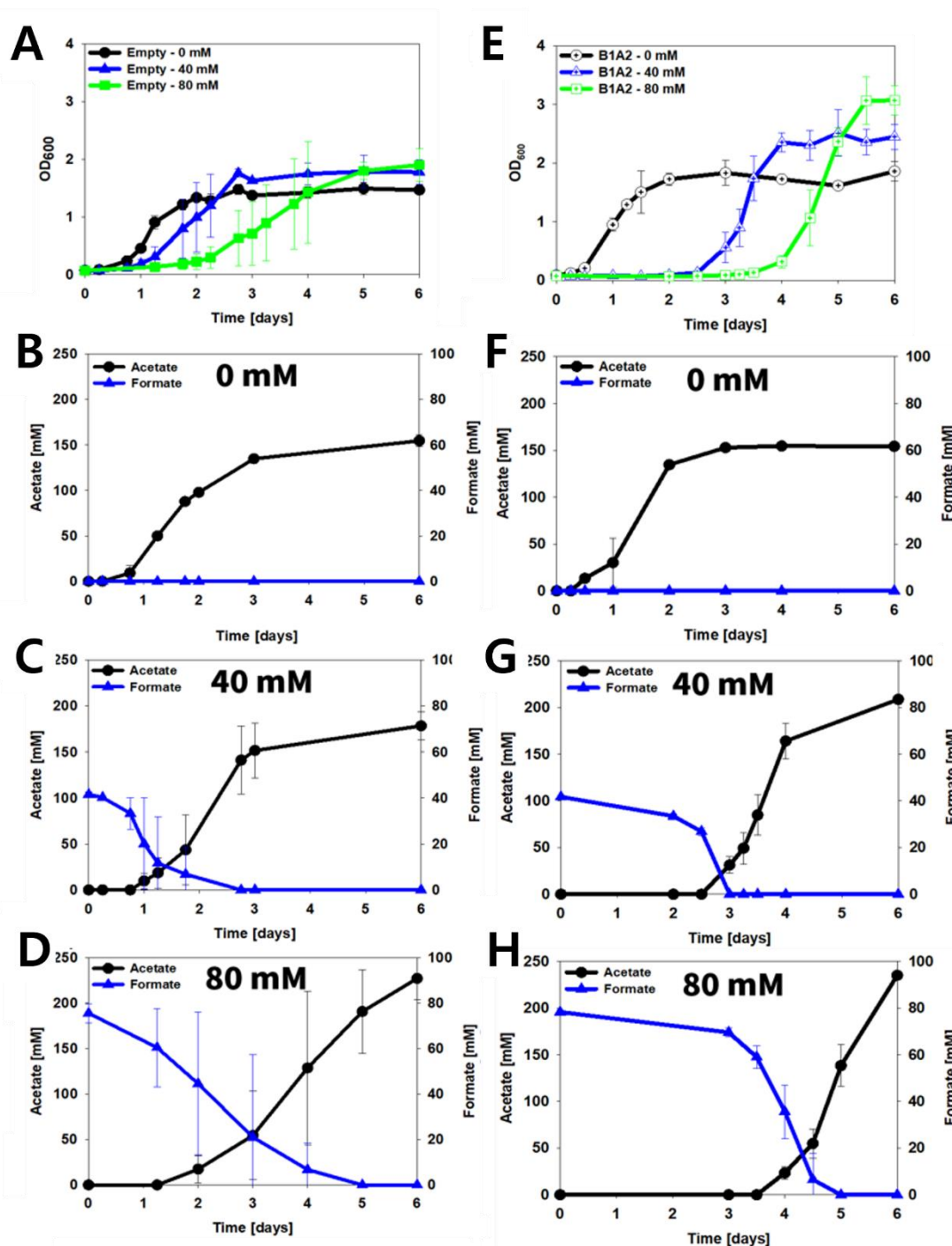
Genome editing provides precise, stable, and inheritable changes, making it well-suited for long-term, targeted applications. While plasmid-based expression is valuable for rapid prototyping and transient needs, genome editing offers superior control, efficiency, and reliability for industrial, medical, and research applications [185]. Several genome editing techniques have been developed for acetogens and *Clostridium* species. Tools such as Clostron, pyrE complementation, allele-coupled exchange, MazF, and CRISPR/Cas (including Cas9 and Cas12a) have been extensively tried in *Clostridium* species [171, 178, 182]. These techniques have proven highly effective for gene deletion. For gene integration, more advanced options, such as a combination of phage serine integrase with CRISPR/Cas9 and transposon-based systems, have been tested, offering new possibilities for efficient and precise gene insertion [190].

## 4.2. Improved substrate utilization and growth

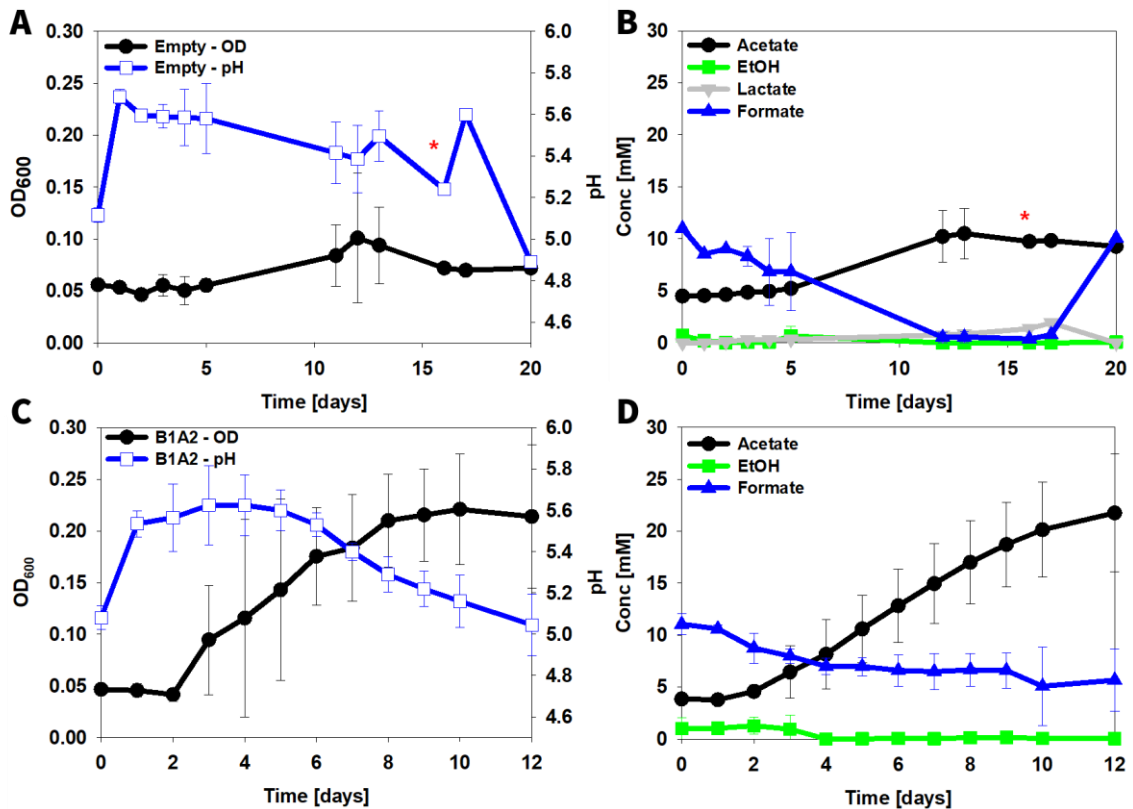
The conversion of pyruvate is a central metabolic process for various pathways [186]. Notably, pyruvate serves as the first intermediate in gluconeogenesis. In *C. ljungdahlii*, pyruvate conversion is catalyzed solely by pyruvate:ferredoxin oxidoreductase, which requires acetyl-CoA, CO<sub>2</sub>, and reduced ferredoxin as substrates. Reduced ferredoxin is a key intracellular electron carrier involved in several enzymatic reactions in acetogens, including ATP synthesis via the Rnf complex, ethanol production through aldehyde:ferredoxin oxidoreductase, the Wood-Ljungdahl pathway, activity of the Nfn complex, and pyruvate synthesis [187]. In contrast, *A. woodii* can also synthesize pyruvate from acetyl-CoA and formate via pyruvate formate lyase [188].

Formate can be readily produced from CO<sub>2</sub> through electrochemical reactions or via MES by acetogens (**Papers II and III**) [31]. In **Paper III**, significant improvements in formate and ethanol production during microbial electrosynthesis were observed after the early stop to acetate production and cell growth caused by evolutionary adaptation to iron. Growth of *C. ljungdahlii* seemed to stop because of a shortage of reduced ferredoxin, which is required for both formate and ethanol production (**Figure 5 in Paper III**). To address this issue, I hypothesized that the re-assimilation of formate via pyruvate formate lyase could enhance *C. ljungdahlii* growth while maintaining ethanol production from CO<sub>2</sub> in a BES (**Papers III and IV**).

In **Paper IV**, heterologous expression of pyruvate formate lyase from *A. woodii* significantly improved cell growth of *C. ljungdahlii* during both gas fermentation and microbial electrosynthesis, with or without formate supplementation, compared to the empty vector control (**Figure 20 and Figure 21**). However, in a BES, formate was not fully utilized by



**Figure 22.** Effect of different sodium formate concentrations on *C. ljungdahlii* expressing pyruvate formate lyase from *A. woodii*. (A–D) Results for the control *C. ljungdahlii* [empty] strain under  $H_2:CO_2$  conditions: (A) OD measurement; and metabolite profiles on (B) 0 mM, (C) 40 mM, and (D) 80 mM sodium formate. (E–H) Results for *C. ljungdahlii* [B1A2] under  $H_2:CO_2$  conditions: (E) OD measurement; and metabolite profiles on (F) 0 mM, (G) 40 mM, and (H) 80 mM sodium formate. Error bars denote standard deviation ( $n = 3$ ). *C. ljungdahlii* [empty] carried the pMTL83121- $P_{fdx}$ -E\_empty plasmid, whereas *C. ljungdahlii* [B1A2] carried the pMTL83121- $P_{fdx}$ -E\_pflB1/pflA2 plasmid. Figure taken from **Paper IV**.



**Figure 23.** Time-dependent behavior of *C. ljungdahlii* expressing pyruvate formate lyase from *A. woodii*. (A, B) Results for *C. ljungdahlii* [empty] grown in a BES reactor: (A) OD and pH; (B) metabolite profile. (C, D) Results for *C. ljungdahlii* [B1A2] grown in a BES reactor: (C) OD and pH; (D) metabolite profile. Error bars denote standard deviation ( $n = 2$ ). Metabolite concentrations are the mean of cathode and anode chambers. Therefore, the initial formate concentration is shown because 20 mM sodium formate was added only to the cathode chamber. *C. ljungdahlii* [empty] carried the pMTL83121- $P_{fdx}$ -E\_empty plasmid; whereas *C. ljungdahlii* [B1A2] carried the pMTL83121- $P_{fdx}$ -E\_pflB/pflA plasmid. Figure taken from **Paper IV**.

the engineered strain. This limitation may arise from the simultaneous consumption and production of formate during MES. A potential solution to this issue would involve the co-expression of a heterologous formate transporter—absent in *C. ljungdahlii*—along with engineered formate dehydrogenases, tailored to meet the requirements of intracellular electron carriers [162].

*C. ljungdahlii* does not naturally grow on formate [23, 189]. However, if it can be engineered to efficiently utilize formate in a BES, this strategy could be integrated with electrochemical formate production using a modified electrode (**section 3.2.2**). Additionally, a methanol-producing electrode could be employed by introducing a methanol assimilation pathway into *C. ljungdahlii* (**section 3.2.2**).



## Chapter 5. Conclusions and Outlook

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### 5.1. Conclusions

In this thesis, CO electro-fermentation using CO-enriched mixed culture and microbial electrosynthesis using *C. ljungdahlii* were investigated. More specifically, a major CO-fermenting bacterium for CO electro-fermentation was identified in **Paper I**. Microbial electrosynthesis and CO electro-fermentation processes were optimized in **Papers I and II**. An ALE approach was taken to improve extracellular electron uptake of *C. ljungdahlii* in a BES (**Paper III**). Introduction of a different pyruvate synthesis pathway addressed low cell growth of *C. ljungdahlii* during gas fermentation and microbial electrosynthesis (**Paper IV**)

#### *Bacterial community analysis supports C. ljungdahlii as a host strain for microbial electrosynthesis and CO electro-fermentation*

In **paper I**, the pre-enrichment step singled out dominant CO-fermenting acetogens in the inoculum, which would then become the major electro-active bacteria in CO electro-fermentation. *C. autoethanogenum* was the dominant species in the CO-enriched mixed culture and its electro-activity was confirmed in a BES. The CO-enriched mixed culture achieved CO electro-fermentation without the help of an exogenous electron mediator, contrasting the results of an earlier study with an unenriched mixed culture. These results indicates that selecting *C. ljungdahlii*, which is electro-active, has high genetic similarity to *C. autoethanogenum*, as the host strain used in this thesis was adequate.

#### *Host-specific process optimization addressed low cell growth and reproducibility problems*

**Papers I and II** tested operational parameters, cathode potential and pH, and culture medium with and without yeast extract. A CO-enriched mixed culture dominated by *C. autoethanogenum* achieved higher ethanol yields in CO electro-fermentation as well as higher cell viability when 10 mA was applied, as opposed to the control without electric current or application of a current of 25 mA (**Paper I**). In **paper II**, Cathode potentials of  $-0.8$  V and  $-1.0$  V vs SHE exhibited optimal coulombic efficiencies (i.e., close to 100%) for microbial electrosynthesis using *C. ljungdahlii* and, in the latter case, also the highest volumetric productivity (higher than at  $-1.2$  V vs SHE). Also, lowering the initial pH of the cathode and anode chambers improved the cell growth of *C. ljungdahlii* in a BES (**Chapter 2**). Finally, supplementation of yeast extract in the culture medium improved reproducibility

of the results (**Paper II**). These results stress the importance of pH management for microbial electrosynthesis and CO electro-fermentation using *C. ljungdahlii*.

***Adaptive laboratory evolution of *C. ljungdahlii* on CO<sub>2</sub> and iron improved formate and ethanol production with decreased cell growth***

Iron as the solid-state only electron donor was used to characterize the extracellular electron uptake mechanism of *C. ljungdahlii* (**Paper III**). However, the adapted *C. ljungdahlii* showed only around 20% acetate production under CO<sub>2</sub> plus iron (**Figure 4** in **Paper III**), slow cell growth in CO<sub>2</sub> plus H<sub>2</sub> (**Table 1** in **Paper III**), and significantly lower cell growth in a BES (**Figure 16**) compared to the wild-type. Whole-genome sequencing results indicate that *C. ljungdahlii* evolved to save available nutrient resources. Interestingly, the evolved *C. ljungdahlii* produced more formate and ethanol than the wild-type in a BES, despite an early halt to cell growth. It is possible that the evolved *C. ljungdahlii* adapted by trying to convert volatile H<sub>2</sub> into intermediates for future recovery by further metabolism.

***Heterologous expression of pyruvate formate lyase in *C. ljungdahlii* improved the cell growth during gas fermentation and microbial electrosynthesis with formate as co-substrate***

In **paper IV**, Pyruvate formate lyase from *A. woodii* was heterologously expressed in *C. ljungdahlii* to improve the cell growth during gas fermentation and CO electro-fermentation. The maximum OD of the engineered *C. ljungdahlii* significantly improved both with and without supplementation of 80 mM sodium formate under H<sub>2</sub>:CO<sub>2</sub> conditions. Supplementation of 80 mM sodium formate prolonged the lag phase; although this could be shortened by pre-adaptation in the same medium. Cell growth and acetate production in a BES were also significantly higher in the engineered *C. ljungdahlii* compared to the control. These results show that genetic engineering help to improve microbial electrosynthesis using *C. ljungdahlii*.

***Development of genetic engineering tools is required for strain development for microbial electrosynthesis and CO electro-fermentation***

Rational engineering of the improved cell growth *C. ljungdahlii* for microbial electrosynthesis was attempted (**Paper IV**). However, the cloning of required genes was challenging due to limited available genetic tools (**Chapter 4**). Expression of pyruvate formate lyase seemed toxic to the cloning host because it was only successful when a low-copy-number vector and a theophiline-responsive riboswitch were used together. Otherwise a mutation on the start codon was observed, abolishing expression. Fine-tuned gene expression is especially important for microbial electrosynthesis due to a possible exaggerated metabolic burden by overexpression of a gene under electron-limited condition



like in a BES. Therefore, more extensive studies are required to develop genetic engineering tools for acetogen.

*The results of my thesis demonstrate that CO electro-fermentation can valorize [CO:CO<sub>2</sub>] gas mixtures while reducing CO<sub>2</sub> emissions.* Pre-enrichment under CO conditions eliminated the need for an exogenous electron mediator and facilitated the identification of the primary bacteria involved in CO electro-fermentation. This work led to optimized operational parameters and strain engineering of acetogens to enhance performance in a BES using *C. ljungdahlii* as a host. These findings highlight key considerations for the development of CO electro-fermentation and microbial electrosynthesis.

*The present thesis also suggests a workflow for the development of CO electro-fermentation process*

A workflow suggested in this thesis can be applied for another BES process development. The workflow included the following steps: i) potential host strain identification (**Paper I**), ii) process optimization (**Paper II**), and iii) strain improvement by evolutionary and genetic engineering (**Papers III and IV**) (**Figure 7**).

## 5.2. Outlook

The immense greenhouse gas emissions resulting from technological advancements are now threatening the stability of our environment and the quality of daily life due to global warming. Addressing this crisis requires innovative and sustainable solutions that not only mitigate emissions but also valorize greenhouse gases into valuable products. Microbial electrosynthesis and CO electro-fermentation stand out as transformative technologies with the potential to contribute significantly to this global effort.

A BES harnesses the fermentation capabilities of electro-active bacteria. Integrating an electrochemical system into a typical fermentation process offers numerous benefits. Electro-fermentation should aim to maximize the synergy between an electrochemical system and a fermentation process. However, several challenges need to be addressed, including the selection and optimization of high-performing host strains, optimization of electrode materials, process optimization, and microbial strain development. As demonstrated in this thesis, additional challenges arise from the application of electrical current during electro-fermentation, such as pH changes in the bioreactor.

CO<sub>2</sub>-based microbial electrosynthesis using acetogens is gaining increasing attention as a method to utilize greenhouse gases for the production of commodity chemicals. However, the technology is still in its early stages. Several challenges need to be addressed, including up-concentration and purification of CO<sub>2</sub> for anaerobic processes, low productivity and titers,

the predominance of low-value acetate as the main product, and the high cost of bioelectrochemical setups. One potential approach is to directly use CO<sub>2</sub> produced from anaerobic fermentation processes for microbial electrosynthesis. Alternatively, CO-containing industrial off-gas (or syngas) can be used for electro-fermentation using CO-fermenting acetogens, aiming to recover waste gases, that are also greenhouse gases, into value-added chemicals.

CO<sub>2</sub>-based microbial electrosynthesis requires high current density to be scalable and economically feasible. The solventogenic acetogen *C. ljungdahlii*, the host strain used in this thesis, is expected to be able to perform CO electro-fermentation based on the finding from **Paper I**. However, the performance of *C. ljungdahlii* in a BES was also inhibited with high current application. Therefore, sustainable ways of pH management should be explored for application of high current density. One approach could involve optimizing separator materials with high proton mobility and low cost. Another option is to employ alkaliphilic acetogens, which have not been extensively studied in the field of gas fermentation. *Alkalibaculum bacchi* is a potential alkaliphilic acetogen candidate for microbial electrosynthesis and CO electro-fermentation, yet its electro-activity has not been tested.

Since it is uncertain whether *A. bacchi* is electro-active, identifying the extracellular electron uptake mechanism in electro-active acetogens is essential. Therefore, identifying hydrogenases with high affinity for H<sub>2</sub> from high-performing acetogens (e.g. *S. ovata*) in a BES should be prioritized. This approach could potentially improve electron uptake or even enable non-electro-active strains to become electro-active through the heterologous expression of high-H<sub>2</sub>-affinity hydrogenases by enabling efficient use of H<sub>2</sub> on the cathode. This also requires extensive studies for the development of genetic tools for acetogens, especially for fine-tuning the expression of several genes to allow expression of multi-subunit enzymes. Such tools would lead to new opportunities for metabolic engineering of acetogens to improve their performance in BESs.

Finally, to maximize the synergy between electrochemical cells and the fermentation process, electrode modification through electroplating with metals such as cobalt (Co), tin (Sn), and copper (Cu) should be tested in a BES. This approach could facilitate electrochemical CO<sub>2</sub> conversion and improve extracellular electron uptake by acetogens, enabling them to utilize electrochemically produced formate and methanol more effectively.

Overall, despite the numerous biotechnological, electrochemical, and material challenges, microbial electrosynthesis and CO electro-fermentation can transform greenhouse gases from a global challenge into raw materials for production of value-added chemicals, contributing to a sustainable future and attainment of international climate goals.

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