Abstract

SANDOVAL ESPINOLA, WALTER JAVIER. A Story of Gases and Electrons: Unveiling *Clostridium beijerinckii* Aerotolerance and its Assimilation of Greenhouse Gas to n-Butanol. (Under the direction of Dr. José M. Bruno-Bárcena.)

The rise of greenhouse gas emissions from fossil fuels has led to undeniable climate change and its repercussions on the biosphere. As a result, research into alternative fuels, such as butanol, has emerged during the last few decades. Anaerobic *Clostridium beijerinckii* is one of the main catalysts for butanol fermentation. However, due to taxonomical confusions, this bacterium was inadvertently cultured under less than optimal conditions, and thus, not allowing the discovery of its real physiological characteristics. Here we discuss the role of gases in *C. beijerinckii* physiology. First, with bench-scale cultures exposed to oxygen, we show for the first time the high aerotolerance of this species. Furthermore, the manganese catalase (Mn-Kat) from *Lactobacillus plantarum* was active when expressed in *C. beijerinckii*, increasing the fitness of the new strain. In this work, we also evaluated the impact of the endogenous fermentation gases (CO₂ and H₂) on the culture performance. In the absence of gas stripping, the total carbon recovered increased from ~50 to ~90%, which is above theoretical heterotrophic values. With this observation in mind, we performed inline monitoring of the gases generated during the fermentation and observed synchronous CO₂ and H₂ oscillations pointing towards their re-assimilation. Further genome and transcriptome analysis revealed the presence and transcription of crucial Wood-Ljungdahl pathway genes. Furthermore, chemostat fermentations sparging different gas compositions revealed the mixotrophic assimilation of syngas (CO, CO₂ and H₂), along with increased carbon recovery above theoretical heterotrophic values. Overall, this is the first evaluation of *C. beijerinckii* demonstrating high aerotolerance and mixotrophic inorganic carbon-capture capabilities.

by
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Dedication

This work is dedicated to all the people who are curious about the universe, and those who pursue science around the world, and especially to those who foster research and education of my home country, Paraguay.
Biography

I was born in Luque, Paraguay in 1986 and attended Colegio Santa Teresita Middle School, and Centro de Capacitacion Tecnica de Luque High School, with an emphasis in electronics. Later, I changed gears and graduated with a Bachelor of Science degree in Biology, from Universidad Nacional de Asuncion in 2010. I worked in the Quality Control department of Agrofrio S.A. meat processing plant for over three years. In 2011, I was awarded with a Fulbright scholarship to continue with my studies as a Master of Science student at North Carolina State University (NCSU), which I received in 2013. Later I was transferred to the Ph.D. program in the same department at NCSU.
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I would like to thank my parents for their support and inspiration for coming to study abroad and to the Fulbright/Itaipu Program for supporting my earlier years in Graduate School. I would also like to thank my mentor Jose M. Bruno-Bárcena, for guiding and inspiring me to be the best among my peers. This research was supported by the College of Life Sciences, the North Carolina Agricultural Research Service, the Biofuels Center of North Carolina, the College of Agriculture and Life Sciences at NC State University, and was partially funded by the USDA Biomass Research and Development Initiative under the award number 2011-10006-30363.
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List of Abbreviations

ABE fermentation: Acetone-Ethanol-Butanol fermentation

RCM: Reinforced Clostridial Medium

HEDB: Previously designed medium by our lab for improved \textit{C. beijerinckii} growth and butanol production

NCIMB: United Kingdom’s National Collection of Industrial, Food and Marine Bacteria

ATCC: American Type Culture Collection

Glu: Glucose

Fruct: Fructose

RCM: Reinforced Clostridial Medium

HPLC: High Performance Liquid Chromatograph

GC: Gas Chromatograph

AP: Apparent Purity

PEP:PTS: Phosnoenolpyruvate-dependent phospho-transpherase system

Hpr: Phosphocarrier protein of the PEP:PTS

CcpA: Catabolite control protein A

CRE: Catabolite Repressor Element

adhe: Alcohol dehydrogenase

ptsk: HPr kinase

P-Ser-HPr: HPr protein phosphorylated at the Serine residue

SpoIIE: Second Stage sporulation protein E

IIBC^{Scr} (\textit{ScrA}): Sucrose-PTS protein

\textit{ScrR}: Transcriptional repressor of the sucrose operon
$ScrB$: Sucrose hydrolase

$ScrK$: ATP-dependent fructokinase

atm: Atmosphere

WL pathway: Wood Ljungdahl pathway
Chapter 1 - Literature Review – *Clostridium beijerinckii*: a very unusual butanol producer

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1.1 Introduction

Over the past few decades, there has been a renaissance in the development of sustainable resources and technologies. This has been accentuated by the undeniable effect of climate change on the anthroposphere (the fraction of Earth built and occupied by humans), first on developing and now on developed countries (hence, the current political interest)\(^1\). This push towards the use of environmentally friendly solutions has had a major effect on the way we generate and maintain our energy security\(^2\).

The "biofuels" era started with the microbial transformation of starch or sugars, mostly from corn or sugarcane into ethanol\(^3,4\). Soon after, this solution became a liability itself: much needed food crops were being depleted in order to satiate the energy needs of rich countries. This led to the development of biofuels generated either from non-edible plant biomass, non-food crops or crops cultivated on marginal lands\(^5\text{–}11\). These were named "second generation" biofuels. At the same time, there was a push for advanced biofuels, specifically, those with higher energy density and other industrially-friendly qualities over ethanol\(^12\). As a result, biobutanol, which is butanol generated through microbial fermentation, has been targeted as an alternative transportation biofuel\(^13\).

Butanol is a four-carbon alcohol that has an array of advantages over ethanol. Key among these properties are: (i) it can be used unblended in unmodified car engines; and (ii) it’s compatible with the current oil infrastructures\(^11\). These characteristics, along with volatile oil prices, have placed butanol among the advanced-biofuels, and whose research has emerged during the past three decades\(^14,15\) (Figure 1-1).
Figure 1-1. Usage of the words “butanol fermentation” in the literature and oil prices in the last 70 years. Percentage of the words “butanol fermentation” among all words in books written in English and published in the United States, between 1930 and 2016. Image generated using Google Books Ngram Viewer (books.google.com/ngrams). Historical monthly West Texas Intermediates (WTI or NYMEX) crude oil prices per barrel from 1946 until June 2016. Oil prices shown are adjusted for inflation using the Consumer Price Index (CPI) and is displayed in log-scale. Data obtained from www.macrotrends.net.

Biobutanol is generated by solventogenic *Clostridium* spp. through their acetone-butanol-ethanol (ABE) pathway. These Gram-positive, anaerobic bacteria, have been employed for decades to generate solvents, as first described by Weizmann in the 1930s\textsuperscript{16}. As with any industrial process, in order to achieve cost-competitive biobutanol production (i.e. by improving butanol production parameters), special interest has been placed in strain development, either through genetic engineering or directed evolution, and the assessment of cheap substrates\textsuperscript{15,17–20}. However, there are still many limitations intrinsic to *Clostridium* physiology and related to culture conditions. For example, due to taxonomic mislabeling, culture conditions have been extrapolated from *C. acetobutylicum* to *C. beijerinckii* (two historical butanol producers), despite the fact that they are very distinct species\textsuperscript{18,21,22}. Indeed, *C. beijerinckii* (which is the focus in this review), is more robust due to a number of features, for example: (i) It has greater flexibility in sugar utilization\textsuperscript{23,24}; (ii) A better process
stability\textsuperscript{25,26}; and, (iii) it’s aerotolerant, which makes it more industry-friendly than the strict anaerobe \textit{C. acetobutylicum} (as will be shown in Chapter 2). Other limitations that have hampered cost-effective butanol production include: (i) The assumption of butanol’s toxicity at relatively low titers; (ii) Sugar utilization capabilities varies between species; (iii) The fermentability of lignocellulosic materials (non-edible plant biomass) is often hindered by the presence of polyphenols, which originate during the hydrolysis steps; (iv) Butanol generation can be unstable, as is the case for \textit{C. acetobutylicum} that contains the relevant genes in a plasmid; and finally (iv) the sporogenic nature of \textit{Clostridium}, as it encounters a nutritional limitation\textsuperscript{7,8,21,27–30}.

Other factors that have been overlooked in ABE-fermentation by \textit{C. beijerinckii} include oxidative stress and the contribution of the gas-phase composition to the fermentation process. Traditionally, \textit{C. beijerinckii} cultures have been started after successfully achieving and maintaining an anaerobic environment. This is normally accomplished by continuously sparging nitrogen gas or by recirculating endogenous H\textsubscript{2} and CO\textsubscript{2}. But this raises the question of what is the impact of these methods, and the gases overall -including oxygen-, on the culture’s performance? For example: (i) Oxygen (which a culture may be transiently exposed to) will consume reducing equivalents (mostly in the form of NADH) for its reduction to water\textsuperscript{30,31}; (ii) Carbon dioxide can serve as an electron sink in some bacteria\textsuperscript{32,33}; and (iii) \textit{C. beijerinckii} fixes nitrogen, which also requires electrons and is energy intensive\textsuperscript{34,35}. At the same time, electrons are needed for solvent and biomass generation\textsuperscript{36,37}. As a result, there is a significant variability in reported product yields, even when comparing the same strains (Figure 1-2). Indeed, we have previously shown higher product yields by \textit{C. beijerinckii} when the endogenous fermentation gases are maintained\textsuperscript{18}, compared to the
values observed when these gases are stripped from the reactor’s head space (Chapter 2 and 3). On the other hand, although nitrogen fixation by solventogenic *Clostridium* is nothing new\textsuperscript{34}, and its relationship with solventogenesis has been previously investigated\textsuperscript{38}, further studies are inviting, for example: (i) Although most media formulations contain a nitrogen source\textsuperscript{39} (which inactivates the nitrogenase complex\textsuperscript{34}), transcriptomic data has shown nitrogen fixation (*nif*) genes overexpression during exponential growth phase\textsuperscript{39}; (ii) With increase ammonium availability, more carbon is assimilated (Appendix 1), indicating nitrogen limitation, and; (iii) The inhibition of the nitrogenase complex leads to increase solvent titers and sugar consumption, also probably due to the channeling of electrons towards products (Appendix 1).

As previously mentioned, much effort has been dedicated to evaluate the fermentability of alternative substrates\textsuperscript{10,40} and to genetic engineer *C. beijerinckii*, while clearly its natural capabilities are not fully understood\textsuperscript{7,18,39,41}. Therefore, there is a need for a deeper understanding of the physiology of this microorganism. Specifically, what role do the electrons play in ABE-fermentation in the context of gas-phase composition?
**Figure 1-2. Total solvents yield variations.** Historical quantifications of total solvents yield (g/g) in batch (black boxes) and continuous cultures (red boxes). Dashed line represents the maximum theoretical value (~0.39 g/g)\(^{42,43}\). Yellow area represents ±10% of the theoretical maximum.

*C. beijerinckii* has historically been considered a heterotrophic strict anaerobic bacterium. Based on latest results regarding the high aerotolerance of this species (Chapter 2) and its capability for mixotrophic gaseous substrate (i.e. inorganic carbon) uptake (Chapter 3), in our view, the traditional approach of solvent fermentation by this bacterium should be reconsidered. In this review, we will focus on the latest findings of the gas phase implications (Figure 1-3) in *Clostridium beijerinckii* cultures in the context of oxidative stress, carbon metabolism, and energy conservation.
Figure 1-3. Overview of three gas stripping profiles during butanol fermentation. Three atmospheres are shown: aerobic, nitrogen and CO₂/H₂ (endogenous or exogenous), with its corresponding active metabolic pathways. In presence of oxygen, the proposed Wood-Ljungdahl (WL) pathway should not active (due to the low CO₂ and H₂ partial pressure), while ROS detoxification pathways should be very active, which leads consumption of redox impacting biomass formation. When N₂ is sparged in the culture the neither the WL (due to CO₂ and H₂ stripping), nor oxidative stress detoxification pathways should be active, which leads to high biomass (compared to aerobic atmosphere). Finally, when endogenous CO₂ and H₂ are retained in the bioreactor, the WL pathway should be active due to the higher CO₂ and H₂ partial pressure, leading to higher total carbon recovered. Additionally, oxidative stress pathways should not be active leading to higher biomass titers (compared to aerobic atmosphere) since both, WL and oxidative stress pathways require reducing power.

1.2 Oxygen reduction and oxidative stress defense mechanisms on C. beijerinckii

Oxygen has a profound impact in biological systems. Not only does it serve as a terminal electron acceptor during energy generation in aerobes, but also, by interacting with cellular elements, it leads to the formation of toxic reactive oxygen species (ROS)²⁹,³¹,⁴⁴–⁴⁶. These ROS (Figure 1:4), such as superoxide anions (O₂⁻) and the highly reactive hydroxyl radicals (OH⁻) (generated through Fenton reactions between H₂O₂ and ferrous ions⁴⁷), along with hydrogen peroxide (H₂O₂), can become lethal by damaging DNA and phospholipids,
surpassing the cell’s repair mechanisms rate\textsuperscript{45}. There is no enzymatic protection against OH\textsuperscript{•}, therefore, most of the cell’s resources are dedicated to prevent its generation by depleting its precursors\textsuperscript{46} (Figure 1-4). Thus, the success of microbes in withstanding oxidative stress depends on their defense mechanisms\textsuperscript{44}. Aerobic bacteria rely on their superoxide dismutase (SOD)/catalase system. SOD catalyzes the dismutation of superoxide anions, either to oxygen and hydrogen peroxide, while catalase reduces the latter molecule into water and oxygen\textsuperscript{48,49}. Conversely, strict anaerobes like Clostridia, do not possess catalase but in some cases may contain a superoxide dismutase or reductase\textsuperscript{29,50–52}. These microbes mainly rely on oxygen-scavenging NADH oxidoreductases, by taking advantage of their reducing equivalents\textsuperscript{53}. These are molecules that transfer the equivalent of one electron in redox reactions, like those involved in oxygen and ROS reduction. In anaerobic bacteria, most of the H\textsubscript{2}O\textsubscript{2} is generated by the interaction of O\textsubscript{2} with flavoproteins and NADH oxidases\textsuperscript{44,54,55}. 


Figure 1-4. Univalent oxygen reduction pathway and generation of reactive oxygen species (ROS). Univalent pathway of oxygen reduction to water requires four electrons and four protons, leading to the formation of ROS intermediates, including superoxide anions (O$_2^-$), hydroxyl radicals (HO•), and hydrogen peroxide (H$_2$O$_2$). Enzymes that reduce each chemical species are shown: oxidases for oxygen, superoxide dismutase (or reductase) for superoxide anion, and catalase / peroxidase for hydrogen peroxide. There is no enzymatic mechanism to detoxify HO•, and its short half-life and high reactivity with macromolecules makes it lethal to cells.

Most of the work regarding oxygen reduction and detoxification mechanisms in solventogenic clostridia has been performed with _C. acetobutylicum_\textsuperscript{29,30,36,56–58}. This information has been adapted into culture conditions (i.e. keeping anaerobiosis) and extrapolated to _C. beijerinckii_ cultures\textsuperscript{28,59,60}. _C. acetobutylicum_ relies on its NADH-dependent desulfoferrodoxin (Dfx) as a superoxide reductase (SOR)\textsuperscript{61}. For defenses against hydrogen peroxide and oxygen, this microorganism relies on its revRbr (reverse ruberythrin), which acts as terminal electron acceptor and it is a component of NADH peroxidase (NADH: H$_2$O$_2$ oxidoreductase) and NADH oxidase (NADH: O$_2$ oxidoreductase) systems, with peroxide as its preferred substrate. For molecular oxygen reduction, two flavodiiron proteins (FDPs), FprA1 and FprA2, are the terminal components of NADH oxidase,
which catalyzes a four-electron reduction of oxygen to water. Here, NADH: rubredoxin oxidoreductase (NROR) or rubredoxin (Rd) are intermediates. Thus, there’s a decrease in reducing power as \textit{C. acetobutylicum} scavenges oxygen and its reduced species. Indeed, \textit{C. acetobutylicum} is a classic example of an obligate anaerobe. This species ceases growth in presence of oxygen until anaerobiosis is reestablished due to an exaustion of its reducing power. Basically, this bacterium depletes its resources to achieve complete oxygen reduction. Not only are ROS toxic for this species, but also molecular oxygen can damage the iron-sulphur center of its pyruvate-ferredoxin oxidoreductase (PFOR), which is central in carbon metabolism.

\textit{C. beijerinckii} also contains Dfx as superoxide reductase and rubrerythrin/rubredoxin-dependent NADH oxidase/peroxidases. In addition, extracts of \textit{C. beijerinckii} have shown SOD activity. Indeed, this species has annotated a superoxide dismutase and reductase in its genome (Cbs_1507, Cbs_1856 and Cbs_3348). Regarding molecular oxygen reduction, to the best of our knowledge, the activities of FprA1 and FprA2 (FprA1/2) have not been experimentally confirmed within this species. However, a BLAST search with FprA1/2 (ID: Q97K92.1 / Q97GC0.1) of \textit{C. acetobutylicum}, turned up a \textit{C. beijerinckii} NCIMB 8052 putative protein (ID: AIU01918) with 43% sequence identity. This protein also has a 99% sequence identity with a flavoprotein in another \textit{C. beijerinckii} strain (ID: WP_026885926). Additionally, this species has the genetic potential to encode an alkyl hydroperoxide / thiol peroxidase, against H$_2$O$_2$ (Cbs_0765, Cbs_2959, Cbs_2977 and Cbs_3035) (Chapter 2). Furthermore, it was recently shown that clostrubins (polyphenols) generated by this species, allow it to grow in oxygenated environments. Although the mechanism for this oxygen reduction pathway is not yet understood, we have shown that \textit{C.
beijerinckii contains an active laccase (Chapter 2). This enzyme performs a four-electron reduction of oxygen to water, in the presence of polyphenols. Thus, this may also be a defence mechanism against oxygen and subsequent oxidative stress in C. beijerinckii.

C. beijerinckii displays an exceptionally high aerotolerance that is not common in strict anaerobes. Indeed, this species is capable of growth even when continuously exposed to oxygen. In this condition, the maximum specific growth rate of C. beijerinckii decreases, as expected, but it does not halt growth (Chapter 2), clearly in contrast with C. acetobutylicum.

1.3 Genetic and enzymatic efforts to combat oxidative stress in solventogenic Clostridia

Most of the efforts to combat oxidative stress in Clostridium cultures have been centered on providing exogenous catalase\textsuperscript{68,69}. Metabolic engineering has also been explored, mostly in C. acetobutylicum, but not C. beijerinckii. Examples of modified C. acetobutylicum include the heterologous expression of a glutathione (GSH) synthetic pathway\textsuperscript{70}; the derepression of ROS-related genes by the inactivation of the regulator PerR\textsuperscript{56}; and recently, the heterologous expression of a water-forming NADH-oxidase (noxE) from Lactococcus lactis\textsuperscript{71}. In the first example, the GSH (which is uncommon in Gram-positive bacteria\textsuperscript{72}), can act as a nucleophile or reductant by serving as cofactor of NADPH-dependent GSH-peroxidases. These, in turn, are important in the defense mechanisms against H\textsubscript{2}O\textsubscript{2} and lipid hydroperoxides\textsuperscript{73}. In the second example, PerR – a member of the ferric uptake regulator (Fur)- is a transcription repressor that senses intracellular H\textsubscript{2}O\textsubscript{2} levels by histidine oxidation\textsuperscript{74}. This protein controls the expression of many genes coding for ROS-scavenging enzymes. Thus, its inactivation in C. acetobutylicum allowed the mutant strain to express
higher levels of rev-ruberythrin, flavoproteins, desulfoferrodoxin and alkyl-hydroperoxidase. Finally, the heterologous NADH-oxidase expression redirected the electrons towards acid generation, but did not increase the fitness of the recombinant strain. Overall, despite the clear metabolic advantages of *C. beijerinckii* mentioned before, there is a lack of studies involving metabolic engineering and herologous gene expression in this species.

The main reason behind the limited literature describing recombinant *C. beijerinckii* strains is the underdeveloped genetic tools for efficient and stable gene expression in this species. Indeed, most of the tools available have been optimized for *C. acetobutylicum*, and due to the historical taxonomic confusion previously discussed, the same approach has been applied to both, but yielding different results: significantly more literature can be found describing genetic engineering in *C. acetobutylicum* when compared to *C. beijerinckii*. The distinctive nature of the replicon appears to be one of the main limitations for successful *C. beijerinckii* transformation. For example, most of the plasmids used for *C. acetobutylicum* have either rolling-circle or theta replicons; however, only theta replicons seem to replicate in *C. beijerinckii*. Interestingly, the streptococcal theta replicon from plasmid pAMβ1 was shown to be stable in *C. beijerinckii* decades ago. Unexplainably, this has been disregarded over the years. Another difference resides in methylation requirements. While *C. acetobutylicum* requires methylated DNA for a successful transformation, *C. beijerinckii* does not. In fact, successful transformation requires plasmids propagation in Dam and Dcm strains, such as *Escherichia coli* ER2925. Recently, our group successfully expressed the manganase-catalase from *Lactobacillus plantarum* in *C. beijerinckii* SA-1. Growing under a constant
flow of air through the head space (overlay), this strain displayed a maximum specific growth rate equivalent to that of wild-type SA-1 cultured anaerobically. Additionally, the catalase increased the fitness of the corresponding strain, under aerobic and anaerobic conditions. To our knowledge, this is the only report of *C. beijerinckii* expressing a heterologous enzyme for hydrogen peroxide protection.

### 1.4 Fermentation gases

In the course of fermentation, as residual oxygen and accompanying ROS are reduced to water by *C. beijerinckii*, the environment experiences a transient change due to the generation of fermentation gases. At this point, H₂ and CO₂ start accumulating in the head space as the culture reaches its exponential growth phase. Hydrogen generation is a mechanism for electron disposal, and is linked to ATP generation⁴². Briefly, a hexose is catabolized to two pyruvates, along with the generation of two ATP and two NADH, each through the Embden-Meyerhof-Parnas (EMP) pathway. Subsequently, pyruvate becomes acetyl-CoA by an oxidative decarboxylation catabolized by pyruvate-ferredoxin oxidoreductase (PFOR), while releasing CO₂. This reaction also leads to a reduced-ferredoxin, which acts as electron donor for the reduction of NAD⁺ to NADH, or the generation of molecular hydrogen by a hydrogenase complex⁸⁶,⁸⁷. Subsequently, acetyl-CoA becomes acetate and butyrate, by the action of acetate and butyrate kinases, respectively. Two more ATPs are generated in this step, along with the reduction of two NADH (for the generation of butyryl-CoA, which leads to butyrate). In a closed system, the concentrations of H₂ and CO₂ reach considerably high levels up to ~ 20 to 40 % (v/v) within the headspace, respectively⁸⁸ (Appendix 2). Interestingly, both gases can also be reassimilated and coupled
to energy-conservation mechanisms\textsuperscript{32,89}. Indeed, in mixotrophic conditions, \textit{C. beijerinckii} SA-1 is able to assimilate both gases, leading to above-theoretical levels of carbon recovery (Chapter 2 and Chapter 3). In later growth phases, as the substrate becomes limiting, the generation of both gases decreases and redox balance is achieved mostly by butanol synthesis\textsuperscript{42}. Clearly, biomass, products, redox balance, and oxygen reduction are linked to NADH availability, which is also involved in energy conservation mechanisms through chemiosmosis\textsuperscript{90}.

1.5 Gaseous substrates

In 1932, Fisher and coworkers opened the door to a new and exciting research field by the discovery that CO\textsubscript{2} can be reduced to acetate in biological processes (or acetogenesis)\textsuperscript{91}. They observed the H\textsubscript{2}-dependent CO\textsubscript{2} reduction to acetate by microbial populations in sewage. Due to the ever-increasing CO\textsubscript{2} and other greenhouse gas emissions and global warming, renewed attention is focused on this metabolic capability. The first bacterium isolated that was able to grow on chemolithoautotrophic substrates, namely H\textsubscript{2} and CO\textsubscript{2} (or CO), was described in 1936 by Wierinka \textit{et al}, and was named \textit{Clostridium aceticum}\textsuperscript{92,93}. However, this microorganism was lost and no further studies were performed with this microbe until the 1980s\textsuperscript{94}, thus, the biochemistry was not really understood at that time. On the other hand, another microbe was later isolated from horse manure by Fontaine and coworkers, also displaying inorganic-carbon assimilation phenotype, and was named \textit{Clostridium thermoaceticum} (currently named \textit{Moorella thermoacetica}, which is one of the most studied acetogens\textsuperscript{95}). This thermophilic, spore-forming bacterium generates 2.5 moles of acetic acid per mole of glucose. Fontaine and coworkers discussed the idea that there must
be a different type of glucose cleavage other than the classical (at the time) 3 – 3 split. In addition, there was no net loss of CO₂, suggesting the generation and later re-absorption of this gas. They called this a “new type of glucose fermentation”⁹⁶. The basic equations (Equations 1 and 2) that describe this phenotype were later demonstrated by Wood and Barker on C. thermoaceticum cultures³²,⁹⁷–⁹⁹. Basically, C. thermoaceticum ferments glucose to acetate, and later, the CO₂ gets re-assimilated to become the same organic acid⁹⁶.

\[
\begin{align*}
C_6H_{12}O_6 + 2H_2O & \rightarrow 2CH_3COOH + 8H + 2CO_2 \quad (\text{Equation 1}) \\
8H + 2CO_2 & \rightarrow CH_3COOH + 2H_2O \quad (\text{Equation 2})
\end{align*}
\]

The metabolic pathway that allows these microbes to re-assimilate CO₂ into acetate is called Wood-Ljungdahl (WL), in honor of the biochemists who first described its enzymology¹⁰⁰,¹⁰¹. This pathway (Figure 1-5) consists of a “linear, one-carbon” synthesis of acetyl-CoA from the reduction of inorganic carbon molecules. Briefly, the protons and CO₂ generated during glycolysis are then re-assimilated into acetyl-CoA in a reductive, linear process, in contrast with other carbon-capture processes that are cyclical, such as Calvin, TCA (tricarboxylic acid cycle) and hydroxypropionate³²,¹⁰⁰. The WL pathway comprises two branches: a carbonyl and a methyl¹⁰²,¹⁰³. The former starts with the reaction catalyzed by the CO dehydrogenase / acetyl-CoA synthase (CODH/ACS), while the latter starts with formate dehydrogenase (unless CO is the substrate, in which case, both branches start with CODH/ACS)⁹⁹. These reactions, especially the one involving the formate dehydrogenase, are thermodynamically unfavorable and require high carbonate or CO₂ concentrations¹⁰². Briefly, in the methyl branch, the formate dehydrogenase reduces CO₂ to formate.
Subsequently, the formyl group is bound to a tetrahydrofolate (THF) by the formyl-THF-synthetase, leading to a formyl-THF. Water is then split off, and the formyl-THF cyclohydrolase generates a methenyl-THF. This, then, gets reduced to methylene-THF, by a methylene-THF dehydrogenase. This molecule is further reduced to methyl-THF by a methylene-THF reductase. Lastly in this branch, the methyl group from the previous molecule gets transferred to a corronoid iron-sulfur protein (CFeSP) via methyltransferases. This methyl group gets subsequently transferred to the CODH/ACS. Simultaneously, in the carbonyl branch the CODH/ACS reduces CO₂ to CO₂. Finally, the CODH/ACS, which is bifunctional, condenses the methyl group from the methyl branch and the CO from the carbonyl branch to acetyl-CoA.

In acetogenic bacteria, acetyl-CoA is the starting point for acetate and corresponding ATP generation. Otherwise, it’s a central molecule that is channeled towards the ABE and acid pathways in *C. beijerinckii*. For example, when this species is cultured without gas stripping (i.e. by keeping its endogenous gases within the fermentation vessel), the total carbon recovered is higher than typical heterotrophic fermentation values, indicating gas re-assimilation (Chapter 2). Furthermore, this species generates extra amounts of butyrate and butanol when cultured mixotrophically in the presence of exogenous syngas (containing CO₂, CO and H₂) (Chapter 3). This suggests that in this species, the carbon/electron flow goes preferentially towards these C-4 compounds, instead of generating more acetate or ethanol. The generation of C-4s instead of C-2s allows for more electrons recycling, since two NADH are oxidized for butyryl-CoA generation. One ATP is generated per mole of butyrate, and two additional NADH are required for butanol synthesis. This observation underscores the importance for the cells to achieve redox balance. Indeed, when acetogens
grow mixotrophically, CO₂ is primarily an electron sink rather than a means for energy conservation. Consequently, exogenous CO₂ may be required for efficient sugar utilization. Interestingly, *C. beijerinckii* also consumes more sugars when cultured in this condition, as a result of the extra electron-sink provided by the inorganic carbon (Chapter 3).

The biotransformation of syngas can also involve the oxidation of CO into CO₂ and H₂, as depicted in Equation 3. In this instance, CO serves as an electron sink when a
microbe is cultured mixotrophically\textsuperscript{89,104,106}. \textit{C. beijerinckii} performs this reaction when exposed to low CO and CO\textsubscript{2} concentrations (~1\% v/v each), due likely to the low affinity of the CODH/ACS to CO\textsubscript{2} (Chapter 3). Furthermore, this species has an annotated gene that codes for a carbonic anhydrase. This would allow for more inorganic carbon bioavailability (Chapter 3).

In addition to its electron-recycling function, other species of the genus \textit{Clostridium} utilize CO to generate acetic acid along with CO\textsubscript{2} and H\textsubscript{2} (Equation 4)\textsuperscript{107,108}.

\begin{align*}
\text{CO} + \text{H}_2\text{O} & \rightarrow \text{CO}_2 + \text{H}_2 \quad \text{(Equation 3)} \\
4\text{CO} + 2\text{H}_2\text{O} & \leftrightarrow 2\text{CO}_2 + \text{CH}_3\text{COO}^- + \text{H}^+ \quad \text{(Equation 4)}
\end{align*}

Over the years, more than 100 species of bacteria were discovered that were capable of inorganic carbon-capture through the WL pathway and most were isolated from soil or animal guts. The majority belong to the genera \textit{Acetobacterium} and \textit{Clostridium}\textsuperscript{32}. Furthermore, this pathway is found in methanogens and sulfate-reducing bacteria\textsuperscript{109}. The discovery of the WL pathway in \textit{C. beijerinckii} is convoluted: first it was discovered that \textit{C. acetobutylicum} displays higher butanol yields when exposed to CO, as a result of electrons re-routing due to the hydrogenase poisoning\textsuperscript{110}. This observation took place before molecular biology allowed for a clear distinction between \textit{C. acetobutylicum} and \textit{C. beijerinckii}. As previously mentioned, since most culturing techniques were developed based on \textit{C. acetobutylicum} physiology and applied to \textit{C. beijerinckii}, this has contributed to the latter’s lack of physiological studies. However, as previously discussed, the variability in product yields reported in the literature (Figure 1-3) points towards overlooked metabolic pathways.
In view of the polyphyletic nature of the WL pathway and the important genetic differences between \textit{C. acetobutylicum} and \textit{C. beijerinckii}, it is not that surprising that the latter is able to assimilate inorganic carbon. Interestingly, this phenotype only holds true under mixotrophic conditions and when CO$_2$ is above 5% (v/v), which allows for above-theoretical heterotrophic carbon recovery (Chapter 3).

1.6 Alternative energy-conservation mechanisms in \textit{C. beijerinckii}

Considering the zero net-energy generated through the WL pathway \textit{per se} (i.e. one ATP is generated by acetate synthesis, and one is spent during the formyl-THF generation), unless acetogens are cultured mixotrophically, they also rely on chemiosmosis for energy conservation$^{89,90,111,112}$. This ion gradient-driven phosphorylation relies on electrons- and protons-transfer reactions that include respiratory chains. The proteins involved in these reactions are proton pumps electron carriers comprised of iron-sulfur proteins (such as ferredoxins), flavoproteins, quinones and cytochromes$^{113}$. The latter, which carries a heme prosthetic group, generates a valence differential by proton translocation$^{114}$. Briefly, the proton gradient generated as the electrons are shuttled through the redox carriers to a terminal electron acceptor drives the ATP synthesis through a F1F0 ATPase. In anaerobes, the electron acceptors are normally nitrate, nitrite, sulfate, sulfur, carbon dioxide, Mn(IV) and Fe(III) oxides, and fumarate$^{89,113,115,116}$. Interestingly, \textit{C. beijerinckii} contains a number of annotated genes coding for proteins belonging to the electron transport chain (ETC) (Chapter 3). Based on the sub-units within the ETC complex, \textit{C. beijerinckii} resembles a facultative organism, rather than a strict anaerobe (Figure 1-6). For example, this species contains genes coding for cytochrome \textit{b5} (Cbs_2439), \textit{c} (Cbs_2960 and Cbs_2976), flavocytochrome \textit{c} (Cbs_2056),
fumarate reductase (Cbs_3779), flavodoxin WrbA (Cbs_3109), NADH: ubiquinone oxidoreductases (Cbs_2992 and Cbs_2450), and NADH: quinone reductase (Cbs_2986/2996). Additionally, C. beijerinckii contains genes coding ferredoxin-NADPH (+) reductases (Cbs_0661, Cbs_1465, and Cbs_2182), NADPH-dependent FMN reductases (Cbs_0273, Cbs_0739, Cbs_2055, Cbs_2211, Cbs_2735, Cbs_3046, Cbs_3354, Cbs_3484, Cbs_3580, Cbs_3737, Cbs_3895, Cbs_4524, Cbs_4585 and Cbs_4970), and the Rnf-complex, as observed in acetogenic autotrophic bacteria^{90} (Chapter 3). Interestingly, it was previously proposed that the Rnf-system (which creates a proton gradient and regenerates NADH while using reduced ferredoxin)^{117} and cytochromes are mutually exclusive among acetogenes^{90}. This contrasts with C. beijerinckii, which contains both systems for energy conservation under mixotrophic conditions (Chapter 3). It is worth remembering that these enzymes are also involved in ROS generation, as previously mentioned.

The cytochrome b5 is a component of cb1, or Complex III of the ETC, which exhibits oxidase activity^{118}. In addition, C. beijerinckii contains genes coding for heme ABC-type transporters (Cbs_0021, Cbs_0679, Cbs_1564, Cbs_3049, Cbs_3123 and Cbs_4146) and heme d1 biosynthesis protein (Cbs_2007/2008), which is involved in nitrite and O$_2$ reduction, although not heme biosynthesis^{119,120}. These genes indicate the potential of this microorganism for a partial respiratory chain, as seen in some lactic acid bacteria (Figure 1-6). For example, facultative anaerobe Lactococcus lactis shows improved growth in aerated conditions when exogenous heme is present in the media composition^{121,122}. Although heme also displays peroxidase activity (thus, protecting the cells^{123}), this molecule is also part of cytochromes, as previously mentioned. While C. beijerinckii has shown nitrite reduction capabilities (Chapter 3), when this bacterium is cultured under the presence of air and
provided with manganese-catalase protection, its biomass yield ($Y_{ATP} \sim 10.5$ g of biomass per mol of ATP$^{124}$) remains within fermentative values (Chapter 2). Thus, in the conditions tested, this does not point towards oxidative phosphorylation for ATP synthesis. Considering that *C. beijerinckii* lacks the genetic potential for heme generation, it would be worth exploring growth conditions in which exogenous heme is provided and test if a partial respiration is possible, similar to *L. lactis*, after controlling for $H_2O_2$ protection. Additionally, since *C. beijerinckii* has not been shown to grow solely on gases (Chapter 3), it is possible that the lack of heme renders the product of these energy-conservation genes inactive, and therefore, does not allow *C. beijerinckii* to grow autotrophically. Altogether, based on the genetic and physiological evidences, the old classification of *C. beijerinckii* in terms of oxygen tolerance and energy conservation seems no longer meaningful, and deserves additional investigation.
Figure 1-6. Comparison of electron transport chain subunits in organisms with diverse aerotolerance. Electron transport chain subunits based on KEGG data, from strict anaerobes (Clostridium acetobutylicum and C. ljungdahlii), microaerophilic (Lactococcus lactis) and facultative (Bacillus subtilis and Escherichia coli). Each block within the ETC units represents the respective sub-units. White means absence and green presence. Golden ribbon on the right indicates oxygen tolerance among the different species presented: top less and bottom more aerotolerant. Dashes represent protons flow.

Clostridium beijerinckii contains elements in common with facultative and strict anaerobic acetogenic species, specifically, NADH dehydrogenase and Rnf-system (which also generates a proton gradient, while regenerating NADH), respectively. Question mark in cytochrome bd indicates that C. beijerinckii has a cytochrome b annotated, which is a component of bd.
1.7 Ecological considerations

Solventogenic clostridial species, such as *C. beijerinckii*, have traditionally been isolated from soil samples\textsuperscript{126}. In this type of environment, this microbe interacts with other organisms, including many heterotrophs, other more “traditional” acetogens and also methanogens\textsuperscript{112}. The latter are more restricted in terms of substrate uptake potential as carbon and energy sources (i.e. mostly confined to CO, H\textsubscript{2}, CO\textsubscript{2}, methanol, formate, methylamines, sulfate and acetate)\textsuperscript{127}; whereas acetogens, in addition to CO, CO\textsubscript{2} and H\textsubscript{2}, also utilize carbohydrates\textsuperscript{112}. On the other hand, most heterotrophs are limited to organic carbon. *C. beijerinckii* has been shown to possess a wide substrate uptake capability, including carbohydrates\textsuperscript{18,39,128–133} and gases (Chapter 3). Among sugars, pentoses and hexoses are particularly relevant, since they constitute most of the plant biomass. *C. beijerinckii* can co-ferment both types of sugars\textsuperscript{39}, allowing it to take advantage of a bigger proportion of the available substrate. Mirroring other acetogens\textsuperscript{112,117}, *C. beijerinckii* thrives in mixotrophic growth conditions, while utilizing CO, CO\textsubscript{2} and H\textsubscript{2} (Chapter 3).

The above-mentioned organisms are mostly strict anaerobes. The high oxygen tolerance of *C. beijerinckii* is a clear advantage of this species over traditional acetogens and methanogens: oxygen diffuses into the soil, which allows the presence of facultative organisms like *Bacillus* spp. or aerobic *Azotobacter* spp. Another consideration is the presence of polyphenols that are known to hinder microbial metabolism\textsuperscript{134–137}. These compounds are the result of plant biomass degradation and are common in the soil\textsuperscript{138,139}. Laccase is one of the main enzymes responsible for degrading these polyphenols\textsuperscript{140–143}. *C. beijerinckii* has the genetic potential to code for this enzyme (Cbs_1124) and has also shown this type of activity (Chapter 2). This observation is noteworthy, considering that the laccase
requires oxygen as co-substrate to degrade polyphenols, adding to the very efficient oxidase activity of \textit{C. beijerinckii}. Interestingly, the generation of clostrubins (i.e. a polyphenol) by some pigmented strains of \textit{C. beijerinckii}, has been shown not only to fight competitors, but also allows this species to colonize oxygenated environments\textsuperscript{67}. These characteristics, plus the ability of \textit{C. beijerinckii} to fix nitrogen\textsuperscript{38,144} and its diverse byproducts (i.e. useful for syntrophs, which depend on interspecies electron transfers\textsuperscript{145}), underscore its relevant place in soil communities (Figure 1-7).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Proposed ecological interactions of \textit{C. beijerinckii} in a soil community. Based on physiological data and its genetic potential, \textit{C. beijerinckii} (shown in the center) interacts with other organisms in anaerobic environments. This type of environment gets transiently exposed to oxygen (allowing the growth or aerobes and the respiration of facultative organisms), which can be reduced to water by the oxidases, laccase and clostrubins of \textit{C. beijerinckii}. Additionally, the sugars derived from plant biomass (including hexoses and pentoses) get bio-transformed by \textit{C. beijerinckii} into products (probably mostly acids, in a free-pH environment\textsuperscript{59}, and also solvents) and gases (\textit{CO}_2 and \textit{H}_2). These products can be used as substrates by methanogens and other syntrophs, and the gases used by both acetogens and methanogens. Moreover, these gases can be re-assimilated by \textit{C. beijerinckii}. Additionally, polyphenols from lignocellulose degradation get oxidized by laccase, helping laccase-negative organisms. Finally, nitrogen fixed from the atmosphere contributes to plant’s growth and clostrubins inhibit the growth of competitors. Yellow arrows indicate external components that interact with \textit{C. beijerinckii}. Blue and golden arrows indicate products and gases, respectively, generated by \textit{C. beijerinckii}. Purple circles represent the different members of a soil community that may interact with \textit{C. beijerinckii}.}
\end{figure}

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1.8 Conclusions

*Clostridium beijerinckii* is one of the traditional butanol producers employed for decades. During recent years, this alcohol has been targeted as a preferred advanced biofuel over bioethanol. Consequently, a lot of effort has been placed on improving butanol titers and productivity, either by assessing different types of substrates, or by metabolic engineering.

In order to improve a fermentation parameter (such as product yields) or an organism, it is imperative to know its limitations and capabilities. Obviously, such limitations will be determined by the culture conditions. *C. beijerinckii*, by virtue of taxonomical confusion with *C. acetobutylicum*, has not been entirely characterized, especially regarding its oxygen tolerance and the impact of the endogenous fermentation gases on its physiology: both mechanisms are linked by the electrons (NADH) powering the respective metabolic pathways. Figure 1-8 shows a metabolic model that places the relevant metabolic components and electron flow in *C. beijerinckii*. Briefly, the reducing equivalents (NADH) generated during glycolysis, power the following metabolic modules: (i) electron transport chain, to generate ATP; (ii) the Wood-Ljungdahl pathway, allowing for inorganic carbon capture and electron sink; (iii) the acetone-butanol-ethanol (ABE) pathway, for ATP generation and electron recycling, and; (iv) the oxygen reduction and oxidative stress detoxification pathways. The occurrence of different modules depends on the atmospheric composition in which *C. beijerinckii* is cultured, thus, having different effects in its physiology, as indicated above.

The recent advances in oxygen tolerance and inorganic carbon capture by *C. beijerinckii* deserve particular consideration. For example, the most accepted definition of acetogens states that these organisms generate acetyl-CoA from CO₂ and H₂ (or CO) through
the WL pathway, while energy conservation is also achieved through chemiosmosis. On the other hand, strict anaerobic bacteria are, by definition, unable to sustain growth in the presence of oxygen. Therefore, we propose that this species should be re-classified as an acetogenic aerotolerant microorganism. Clearly, this contrasts with other acetogens and industrial solvent producers, placing *C. beijerinckii* in a unique place, while underscoring the polyphyletic nature of the WL pathway. Indeed, many questions arise from these new observations: for example, which enzymes provide the remarkable oxygenase activity of *C. beijerinckii*? Is the cytochrome *b* functional? What proportion of exogenous CO₂ (and CO) is incorporated into the extra products generated? What are the biochemical characteristics of the laccase in *C. beijerinckii* and how does it compare to others? What’s the mechanism of clostrubins against ROS? Is it related to the laccase? Does the laccase have lignolytic activity? What’s the activity of the carbonic anhydrase in *C. beijerinckii*? Clearly, after almost a century of studies with solventogenic Clostridia, *C. beijerinckii* remains a target that invites further investigation.
Figure 1-8. Model showing interactions between carbon metabolism, energy conservation, and oxygen reduction/detoxification, with NADH as nodal point. Glucose (or alternative hexoses) enter the cell through PEP-PTS system (phosphoenolpyruvate-dependent phosphor-transferase)\(^{133}\), and generates (per mol) 2 NADH, 2 ATP (through substrate level phosphorylation) and pyruvate. The latter becomes acetyl-CoA and CO\(_2\). Simultaneously, at the nodal center, NADH is involved in ROS detoxification, as it is required by NADH-dependent oxygenases, peroxidases and SOD/R. Additionally, NADH is involved in CO\(_2\) evolution through ferredoxin and hydrogenases (reaction between pyruvate and acetyl-CoA). The electron transport chain also requires NADH for pumping protons and the subsequent generation of ATP through chemiosmosis. NADH is also required in solventogenesis and carbon assimilation in the ABE and WL pathways, respectively. Other enzymes are also shown, including: hydrogen uptake, for additional reducing power; carbonic anhydrase, for extra CO\(_2\) assimilation; and FMN and nitrite reductases, also involved in energy conservation\(^{90}\).
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Chapter 2 - The aerotolerant phenotype of *Clostridium beijerinckii* and heterologous expression of a non-heme catalase

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2.1 Abstract

Biobutanol generated by solventogenic *Clostridium* is once again being targeted as an alternative advanced-biofuel. However, many limitations hinder *Clostridium* proliferation, including tolerance to oxygen exposure and oxidative stress. Members of the Clostridia group are considered strict anaerobes, mostly due to traditional cultivation methods arising from a one-size-fits-all approach, even among genetically unrelated species. Here, we report a genetic tool for stable heterologous gene expression in *C. beijerinckii* and a comparative study of the impact of oxygen on the growth of *C. beijerinckii* SA-1N and SA-1K; two new strains carrying the same replicative vector. Singularly, SA-1K actively expresses the well-characterized manganese-catalase (MnKat) from *Lactobacillus plantarum*. The growth kinetics under oxygen exposure demonstrate that the new strain carrying the active MnKat (SA-1K) was able to grow at comparable rates to that of SA-1 under anaerobiosis, while increasing biomass accumulation at the expense of products. Surprisingly, mid-log SA-1N cultures (MnKat−) were also able to tolerate continuous exposure to oxygen. Additional bench-scale experiments culturing these new strains allowed for the evaluation of the impact of stripping the fermentation gases from the headspace (CO₂ and H₂) on yield parameters. Overall, this work provides evidence of the natural aerotolerance of *C. beijerinckii* whose fitness can be improved by the heterologous expression of an active manganese-catalase.

2.2 Significance

Climate change is one of the most urgent difficulties facing mankind and research towards renewable resources, such as butanol, is experiencing an upward trend. Butanol is generated by the solventogenic *Clostridium beijerinckii*. However, its physiology under
microaerophilic conditions has not been evaluated. We discovered that *C. beijerinckii* is able to grow and generate products in the presence of oxygen; contrary to how it was traditionally handled. We have also expressed an active manganese-catalase into *C. beijerinckii*, allowing it to match the maximum specific growth rate of the non-catalase-strain under anaerobic conditions, while duplicating the biomass concentration. Our work offers a genetic tool that allows for stable heterologous gene expression in *C. beijerinckii*, while increasing our understanding of its physiology.

### 2.3 Introduction

The increasing greenhouse gas emissions from fossil fuels, and its impact on the biosphere, has reinvigorated efforts on renewable energy research\(^1\)–\(^4\). Accordingly, (bio)butanol is being evaluated as a preferred advanced-biofuel due to its benefits over bioethanol. For example, butanol contains higher energy density and can be used by unmodified car engines, while being compatible with current oil infrastructure\(^2\),\(^5\)–\(^9\). Solventogenic *Clostridium* species, such as *C. beijerinckii*, are the main catalysts for (bio)butanol generation\(^10\)–\(^14\). However, there are many challenges associated with microbial solvent production, hindering biomass and product accumulation\(^12\). Traditional approaches used to overcome these limitations has involved the understanding of environmental stressors and attempting to control their impact on butanol production\(^15\)–\(^22\). Moreover, oxygen exposure and oxidative-stress tolerance has been traditionally overlooked in this taxonomic group, although solventogenic Clostridia are considered obligate anaerobes.

The complete reduction of oxygen to water requires four electrons and four protons and follows a univalent pathway, which paradoxically leads to the formation of partially
reduced reactive-oxygen-species (ROS). These partially-reduced molecules include superoxide anion \( (O_2^-) (+_1 e^-) \), and the highly reactive hydroxyl radical \( (\cdot HO) (+_3e^- + 3H^+ \text{ under } Fe^{2+}) \), which arises through Fenton reactions between hydrogen peroxide \( (H_2O_2) (+_2e^- + 2H^+) \) and ferrous ions\textsuperscript{23}. Hydrogen peroxide, in turn, accumulates in the cells by the interaction between \( O_2 \) and flavoproteins and spontaneous superoxide anions dismutation\textsuperscript{24}. Ultimately, these radicals, specially \( \cdot HO \), results in cellular oxidative stress and damage of DNA, proteins and phospholipids, leading to cell death\textsuperscript{25}.

The success of microbes to withstand oxidative stress often depends on their defense mechanisms, which is centered in preventing the generation of \( \cdot HO \) by detoxifying its precursors\textsuperscript{26,27}. For example, aerobic microorganisms protect themselves from some of these molecules through the superoxide dismutase (SOD)/catalase system. SOD catalyzes the dismutation of \( O_2^- \) either to \( O_2 \) or \( H_2O_2 \), while catalase reduces \( H_2O_2 \) to water and oxygen\textsuperscript{24,28}. While strict anaerobes generally do not possess catalase, it is more frequent the presence of superoxide dismutases or reductases\textsuperscript{29–32}. Thereby, to detoxify \( H_2O_2 \), anaerobes and microaerophilic organisms rely mostly on NADH-dependent oxidases/peroxidases\textsuperscript{33}. However, the use of reducing power (i.e. NADH) to detoxify \( H_2O_2 \), impacts negatively cell growth and products accumulation\textsuperscript{34}. In contrast, catalases do not require reducing equivalents, making them more advantageous over peroxidases.

Catalases are traditionally grouped based on their catalytic redox cofactor: heme and non-heme manganese-catalase\textsuperscript{35}. The latter corresponds to the minority and has only been characterized from \textit{Lactobacillus plantarum}\textsuperscript{36–38}, four other thermophilic bacteria\textsuperscript{39–43}, and \textit{Salmonella enterica}\textsuperscript{44}. 

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The manganese-catalase from *L. plantarum* is composed of six subunits, each containing two manganese atoms at their active center, as opposed to heme. The corresponding gene (*MnKat*) has been expressed in *E. coli*, but the enzyme was inactive and accumulated as inclusion bodies. Additionally, *MnKat* has been previously expressed under the control of its original promoter in a limited number of lactic acid bacteria (LAB) members. However, it only showed activity in *Lactobacillus casei*, probably due to its intracellular manganese levels. Alternatively, heme-catalases have also been expressed in other LAB, but those had the disadvantage of requiring the addition of exogenous heme to the medium, since these bacteria cannot synthesize this prosthetic group.

Among solventogenic Clostridia, the strict anaerobe *C. acetobutylicum* has been the main subject of reports regarding endogenous oxidative stress defense mechanisms. However, taxonomic mislabeling between this species and *C. beijerinckii*, has led to a generalization of growth conditions, while their physiology and genetics differ significantly. For example, an anaerobic environment is traditionally established and maintained during solventogenic fermentations, which is required for strict anaerobe *C. acetobutylicum*. This resulted in the limited understanding of the impact of microaerophilic culturing conditions on growth and products generation by *C. beijerinckii*. Additionally, compared to *C. acetobutylicum*, the genetic tools for this species are relatively underdeveloped. For instance, to our knowledge, there is no example in the literature showing heterologous expression of oxidative stress related enzymes in *C. beijerinckii*.

Indirect and direct evidence from transcriptomic and proteomic analysis suggest that *C. beijerinckii* carries a large array of oxidative stress defense mechanisms, commonly found in aerotolerant microbes. For example, *C. beijerinckii* may contain active ruberythrin (Rbr) /
rubredoxin (Rd) - dependent NADH oxidase/peroxidases and glutathione peroxidase, as well as thioredoxin reductase, able to reduce disulfide bonds in proteins oxidized by \( \text{H}_2\text{O}_2 \)\textsuperscript{58–60}. Additionally, cell free extracts of \( C. \text{beijerinckii} \) have shown SOD activity (i.e. protection against superoxide radicals)\textsuperscript{61}. Furthermore, although not in the context of solvent fermentation, pigmented strains of \( C. \text{beijerinckii} \) has been shown to tolerate oxygenated environments by the generation of clostrubins, which is a polyphenol\textsuperscript{62}. While the mechanism is not yet clear in \( \text{Clostridium} \) spp, an oxygen-consuming enzyme that also targets polyphenols may be involved, such as an active laccase\textsuperscript{63}.

In this study, first such example in \( C. \text{beijerinckii} \), we achieve stable heterologous gene expression allowing for the evaluation and unveiling of the natural aerotolerance of this industrial butanol-producer. Thus, let us to prove a fitness improvement when comparing the kinetic and growth parameters of the wild type to those of a new strain expressing actively the manganese-catalase (\( \text{MnKat} \)) from \( \text{Lactobacillus plantarum} \)\textsuperscript{45}.

### 2.4 Results

#### 2.4.1 Transformation and expression of an active manganese-catalase (\( \text{MnKat} \)) in \( C. \text{beijerinckii} \) SA-1

\( \text{Clostridium beijerinckii} \) SA-1 is an solventogenic hyper-producing strain overlooked for decades and cultured as a typical anaerobe\textsuperscript{56}. In order to generate a more industrially-friendly strain, we expressed the manganese-catalase (\( \text{MnKat} \)) gene from \( \text{Lactobacillus plantarum} \) ATCC 14431\textsuperscript{45} into SA-1. This gene was cloned into the plasmid pTRKH\textsubscript{2}\textsuperscript{64}, and propagated in \( E. \text{coli} \) ER2925 (Dam- and Dcm-)\textsuperscript{65}, as described in Materials and Methods. We have also tested other \( E. \text{coli} \) strains, but only those coming from ER2925, which
generates un-methylated DNA, were recoverable in SA-1. This contrasts with C. acetobutylicum, which requires methylated plasmids for transformation. For the catalase expression in C. beijerinckii, we used the ferredoxin promoter from C. pasteurianum, along with the replicon from pAMβ1 (from Enterococcus faecalis). The pTRKH2 plasmid carrying MnKat, plus the empty vector (used as control), were electroporated into C. beijerinckii SA-1 yielding a time constant of 3.9 ms. The transformation frequencies were $1.8 \times 10^5$ and $3 \times 10^3$ CFU/mL, for the catalase-containing plasmid (SA-1K) and for the control (SA-1N), respectively. Colony PCR amplifications of the erythromycin and catalase genes, and the empty and whole plasmids, revealed the expected size for each product ($\approx 0.5, 1, 7$ and 8 kb, respectively). To corroborate that we were working with the strain SA-1, we also PCR-amplified its unique insertion sequence. In addition, we used total DNA from the mutant SA-1 strains and transformed E. coli ER2925 recovering the intact intended vectors, all of which validated the presence of the plasmids in the transformats SA-1K and SA-1N strains.

To evaluate the expression of an active catalase, we measured its activity on cell-free extracts (CFEs) from SA-1K (MnKat$^+$) and SA-1N (MnKat$^-$). This was calculated to be 3.1 U/g of protein of SA-1K. As expected, there was no catalase activity in CFE from SA-1N. Additionally, the sample maintained its activity after one week of storage at 4 °C.

### 2.4.2 Determination of volumetric oxygen transfer coefficients and dissolved oxygen evolution

In order to obtain a baseline for dissolved oxygen (DO) kinetics, we oxygenated bench-scale reactors containing only medium, by flowing air ($F = 12.48$ L/h) through a
headspace overlay. The soluble oxygen evolution to the medium was monitored using an in-line dissolved oxygen (DO) probe and the slopes of the lines were used to estimate oxygen mass transfer rates (K_{L_a}), or volumetric oxygen transfer coefficient calculated as a non-fermentative model\(^{69}\) (Figure 2-1). The K_{L_a} values for overlay with agitation (our experimental condition) and sparging were closer than the values with overlay with no agitation (Figure 2-1).

\[ \text{Figure 2-1. } K_{L_a} \text{ determination using a non-fermentative model } \frac{dC}{dt} = K_{L_a} (C^* - C_L)^6. \]

Defined medium\(^{15}\), with 6% (w/v) sucrose as carbon and energy source at 37°C, pH: 6.5. Agitation set at 250 rpm and volume: 1400 mL. The dissolved oxygen (DO) was continuously monitored by an in-line INGOLD dissolved oxygen probe (Mettler Toledo, OH, USA). Once dissolved oxygen reached 0% (v/v) by sterile nitrogen sparging, air was flowed steadily at 12.48 L/h. DO was measured in media without cells and air was input by: (circles) sparging, (squares) overlay (headspace) and (triangle) disturbed overlay by stirring the liquid/gas interphase with the agitation blades. Additionally, in media with cells at OD_{600nm} = 0.1 by (black stars) with disturbed overlay.
2.4.3 Impact of oxygen on C. beijerinckii SA-1K and SA-1N cultures proliferation and dissolved oxygen accumulation

We then evaluated the impact of oxygen on the proliferation of C. beijerinckii SA-1K (MnKat+) and SA-1N (MnKat-), and the DO evolution under the conditions described above. Interestingly, under saturated oxygen levels (DO = 100%) and low biomass (OD_{600} \sim 0.1), SA-1K failed to begin proliferation (Figure 2-2 - A). Nonetheless, the oxygen accumulated at a rate similar to that of the uncultured media with undisturbed air overlay (K_{La} = 1.11 \text{ h}^{-1}). Therefore, in order to initiate the fermentation, we maintained anaerobiosis at inoculation by sparging nitrogen gas (Figure 2-2 - A1). Once OD_{600nm} reached 1, the air flow through the overlay setting was initiated (Figure 2-2 – A2 – B1). Strain SA-1K was able to maintain dissolved oxygen values at 0% throughout the fermentation, with no impact on maximum specific growth rate. As expected, when sugar was depleted and sporulation initiated, DO increased abruptly to saturation. Interestingly, in SA-1N cultures (MnKat-), DO transiently reached 8%, and decreased to 0% after 20 h of culture, coinciding with maximum biomass titers. At sporulation, the DO dynamic showed the same kinetics as with SA-1K (Figure 2-2 – B1).
Figure 2-2. Bench-scale batch growth experiments of *C. beijerinckii* SA-1K and SA-1N strains (A and B, respectively) showing evolution of dissolved oxygen and biomass. A. Culture of *C. beijerinckii* SA-1K was initiated and exposed to oxygen by a continuous flow of air thru the head space (overlay) at A1: Anaerobiosis was reestablished by sparging sterile N₂ gas and maintained until A2: When optical density reached one (OD₆₀₀nm ~ 1), continuous flow of air thru overlay was resumed. B: Culture of *C. beijerinckii* SA-1N was initiated under anaerobic conditions. B. When optical density reached (OD₆₀₀nm) ~ 1 the continuous flow of air thru the overlay was initiated. Defined medium [15] containing 6% (w/v) sucrose as limiting carbon and energy source at 37°C, pH: 6.5. Agitation set at 250 rpm. Volume: 1400 mL. Constant airflow was always at 12.48 L/h.

2.4.4 Impact of oxygen exposure on the kinetic and yield parameters of *C. beijerinckii* SA-1K and SA-1N

Under oxygen exposure, the kinetics and titers of biomass and products differed between both strains: SA-1K displayed a 24% faster maximum specific growth rate (μₘₐₓ) and generated 25% more biomass than SA-1N, along with 1 g/L more acetate. Moreover, SA-1K μₘₐₓ (under oxygen exposure) was comparable to that of SA-1 (wild-type) cultured anaerobically (Table 2-1). Conversely, SA-1N generated 10 and 11% more butanol and total solvents, respectively, compared to SA-1K (Figure 2-3 and Table 2-1). Both strains, SA-1K and SA-1N, displayed simultaneous solventogenesis and acidogenesis, while SA-1N reassimilated more butyrate into butanol.

We also evaluated the carbon recovery under the conditions described above (with the headspace air overlay stripping the endogenous fermentation gases [CO₂ and H₂]), and with
no air overlay (with the fermentation gases maintained within the reactor). C-mol yields showed higher carbon recovery (~ 90%) when the endogenous gases (CO₂ and H₂) were not stripped, regardless of the strain (Table 2-1). However, with an air overlay, a total ~ 50% of the total carbon was recovered, which is closer to theoretical heterotrophic values.

Figure 2-3. Bench-scale batch culture experiments showing product accumulation and sugar consumption by C. beijerinckii SA-1K and SA-1N. Product accumulation and sugar consumption by Clostridium beijerinckii SA-1K (A) and SA-1N (B) cultured with air overlay at 12.48 L/h, once OD₆₀₀nm reached 1. Bench-scale batch fermentations in defined medium [15], with 6% (w/v) sucrose as limiting carbon and energy source at 37°C, pH: 6.5. Agitation set at 250 rpm. Volume: 1400 mL.

2.4.5 Determination of plasmid stability

In order to test for plasmid stability, we performed each bench-scale experiment without erythromycin (i.e. the plasmid selective marker). We took samples at the beginning and end of each fermentation and plated on M17 agar plates containing or not 5 µg/mL erythromycin. The results highlight the plasmids’ stability by showing ~ 90% of colonies retaining erythromycin resistance.
2.4.6 Cultures challenged to ferulic acid and laccase activity

Plant biomass is currently being evaluated as a substrate for 2nd generation biofuels. However, in order to obtain its free sugars, it must first endure pretreatments that also release phenolic compounds, including ferulic acid. Although this compound provides antioxidant activity, it also hinders ABE fermentation in concentrations as low as 0.3 g/L. The addition of laccase to this type of substrate has been shown to mitigate the effects of free phenolic compounds, while in certain conditions, increasing the antioxidant activity of ferulic acid. Additionally, transcriptomic data has indicated the expression of oxidative stress related genes when *C. beijerinckii* is cultured in the presence of ferulic acid. Therefore, we evaluated the impact of increasing concentrations of ferulic acid on microaerobic (and anaerobic) cultures of SA-1K (MnKat+) and SA-1N (MnKat-) (Figure 2-4). Under microaerobic conditions, SA-1K tolerated up to 0.3 g/L ferulic acid, whereas SA-1N only up to 0.1 g/L. Interestingly, neither strain grew without the addition of ferulic acid. On the other hand, under anaerobic conditions, SA-1K generated 3X more biomass than SA-1N when both strains were grown in the presence of up to 0.3 g/L ferulic acid, consistent with its increased fitness phenotype. Additionally, both strains were able to proliferate at concentrations up to 1 g/L of ferulic acid. The difference in tolerance between microaerobic and anaerobic conditions, suggests further oxidative stress limitations between 0.3 and 1 g/L ferulic acid.

*C. beijerinckii* has the genetic potential to encode for a laccase (Cbs_1124), which consumes oxygen while oxidizing phenolic compounds. In order to test if this gene coded for an active enzyme, we measured its activity on CFEs of *C. beijerinckii*. We determined
4.64 ± 1.28 U/mg of protein and after four days at 4 °C, its maintained 81% of its activity. To our knowledge, this is the first report of an active laccase in a *Clostridium* spp.

**Figure 2-4. Final biomass concentration of *C. beijerinckii* cultured in the presence of ferulic acid. *C. beijerinckii* was cultured in defined media containing 30 g/L sucrose, 4 μg/mL erythromycin and 1mM MnCl₂ at 37 °C, anaerobically (black symbols) or aerobically (open symbols). Ferulic acid was added at the time of inoculation, at different concentrations. Each culture was started at an OD₆₀₀nm of ~ 0.1. Values shown indicate final optical density (OD₆₀₀nm) at the end of the fermentation. Values below red dashes indicate no growth.
Table 2-1. Kinetic and yield parameters from batch cultures of *C. beijerinckii* SA-1, SA-1N (empty vector) and SA-1K (*MnKat*), cultured with or without constant air flow through the head space (overlay). Defined medium\(^{12}\), 6% (w/v) sucrose as limiting carbon and energy source at 37°C, pH: 6.5. Agitation set at 250 rpm. Volume: 1400 mL. Air constant flow was initiated at 12.48 L/h through the head-space once OD\(_{600nm}\) reached 1. ± Indicates standard deviation.

<table>
<thead>
<tr>
<th>Kinetic and Yield Parameters</th>
<th>SA-1N</th>
<th>SA-1K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass (OD600/mL)</td>
<td>8.6</td>
<td>11.4</td>
</tr>
<tr>
<td>Biomass yield (OD600/g)</td>
<td>0.14</td>
<td>0.19</td>
</tr>
<tr>
<td>Butanol (g/L)</td>
<td>9.30 ± 0.03</td>
<td>8.43 ± 0.03</td>
</tr>
<tr>
<td>Acetone (g/L)</td>
<td>4.11 ± 0.09</td>
<td>3.87 ± 0.03</td>
</tr>
<tr>
<td>Ethanol (g/L)</td>
<td>0.47 ± 0.06</td>
<td>0.39 ± 0.02</td>
</tr>
<tr>
<td>Total Solvents (g/L)</td>
<td>13.79 ± 0.03</td>
<td>12.32 ± 0.03</td>
</tr>
<tr>
<td>Specific Growth rate (\mu) (h(^{-1}))</td>
<td>0.27</td>
<td>0.39</td>
</tr>
<tr>
<td>Butanol yield (Yg/g)</td>
<td>0.15</td>
<td>0.14</td>
</tr>
<tr>
<td>Total Solvents yield (Yg/g)</td>
<td>0.22</td>
<td>0.21</td>
</tr>
<tr>
<td>Butanol productivity (g/L.h)</td>
<td>0.2</td>
<td>0.21</td>
</tr>
<tr>
<td>Total carbon recovered</td>
<td>0.47</td>
<td>0.49</td>
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<table>
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<th>Kinetic and Yield Parameters</th>
<th>SA-1</th>
<th>SA-1K</th>
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<td>12.58</td>
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<td>Biomass yield (OD600/g)</td>
<td>0.1</td>
<td>0.19</td>
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<tr>
<td>Butanol (g/L)</td>
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<td>13.32 ± 0.36</td>
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<tr>
<td>Acetone (g/L)</td>
<td>11.22 ± 0.04</td>
<td>6.06 ± 0.05</td>
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<tr>
<td>Ethanol (g/L)</td>
<td>0.59 ± 0.01</td>
<td>0.12 ± 0.02</td>
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<tr>
<td>Total Solvents (g/L)</td>
<td>25.30 ± 0.04</td>
<td>19.58 ± 0.21</td>
</tr>
<tr>
<td>Specific Growth rate (\mu) (h(^{-1}))</td>
<td>0.39</td>
<td>0.39</td>
</tr>
<tr>
<td>Butanol yield (Yg/g)</td>
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<td>0.31</td>
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<tr>
<td>Total Solvents yield (Yg/g)</td>
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<td>0.46</td>
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<tr>
<td>Butanol productivity (g/L.h)</td>
<td>0.34</td>
<td>0.33</td>
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<tr>
<td>Total carbon recovered</td>
<td>0.77</td>
<td>0.9</td>
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2.5 Discussion

The determination of factors that limit growth and product generation within solvent-production systems is imperative for cost-competitive processes. These types of fermentations are performed by strictly anaerobic solventogenic *Clostridium* species, but transient oxygen exposure and the consequent oxidative stress is a constant element of concern. Considering that *C. beijerinckii* does not contain the genetic potential for heme biosynthesis, we transformed this microorganism with a replicative plasmid containing a manganese-catalase from *L. plantarum*. The transformation efficiency differed between plasmid with or without catalase by 2 logs, pointing towards the protective properties of catalase in maximizing recoverable colonies. In fact, this protection was also observed at bench-scale levels, in cultures exposed to oxygen. Here, SA-1K (*MnKat*⁺) accumulated biomass close to energetic theoretical levels⁷⁵,⁷⁶ (Table 2-2), grew faster than SA-1N (*MnKat*⁻), and matched the maximum specific growth rate (µ<sub>max</sub>) of SA-1 grown anaerobically, clearly demonstrating catalase protection. Surprisingly, while at a reduced specific growth rate compared, SA-1N was also able to grow and generate products, while keeping dissolved oxygen below 10%. The abrupt dissolved oxygen accumulation after sugar limitation and consequent sporulation, underscores the strength of this species to handle oxygen when metabolically active.
Table 2-2. Total energetic biomass yields from sucrose to products. Energetic biomass yields obtained from experimental values of biomass and products of *C. beijerinckii* SA-1, SA-1N (empty vector) and SA-1K (MnKat+), cultured with or without a constant airflow through the head-space (overlay). ATP values were calculated based on amount of ATP per products on each condition. Theoretical biomass per amount of theoretical ATP: Y_{ATP} \sim 10.5 \text{ g biomass/mol ATP}. Defined medium 15%, 6% (w/v) sucrose as limiting carbon and energy source at 37°C, pH: 6.5. Agitation set at 250 rpm. Volume: 1400 mL. Air constant flow was initiated at 12.48 L/h through the head-space once OD_{600nm} reached 1.

<table>
<thead>
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<th>Parameters</th>
<th>Air overlay/gas stripping</th>
<th>No air/No gas stripping</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SA-1N</td>
<td>SA-1</td>
</tr>
<tr>
<td>ATP (mol)</td>
<td>0.4753752</td>
<td>0.505623</td>
</tr>
<tr>
<td>Biomass (g/L)</td>
<td>4.382084</td>
<td>5.217016</td>
</tr>
<tr>
<td>Y_{ATP} (g Biomass/mol ATP)</td>
<td>9.2181595</td>
<td>10.31799</td>
</tr>
</tbody>
</table>

Our experiment with ferulic acid shows that SA-1K tolerates higher concentrations of this phenolic compound in aerated conditions. This indicates the presence of H$_2$O$_2$ and further physiological evidence of the catalase protection. Interestingly, under anaerobic conditions both strains (SA-1K and SA-1N) grew in the presence of higher concentration of ferulic acid. This indicates that aerobically another limitation arises, perhaps H$_2$O$_2$ generation (and ROS) that exceeds its reduction. Overall, the increase ferulic acid tolerance by SA-1K can be advantageous for the production of 2nd generation biofuels that rely on plant biomass.

The lack of growth in aerobic conditions without ferulic acid may be due to the antioxidant and free-radicals scavenging activity provided by this polyphenol, specially, since laccase may modify this molecule to increase its antioxidant activity$^{72}$. Furthermore, we confirmed the presence of a laccase in *C. beijerinckii*. To our knowledge, this is the first such report in *Clostridium*. However, the role of this enzyme in the above-mentioned phenotype deserves further investigation.

The catalase expression also allowed for the evaluation of endogenous fermentation gases (i.e. CO$_2$ and H$_2$) and its impact on the culture. When they were stripped from the
broth, only the sugar-derived carbon was recovered (~ 50%). However, without air overlay, the endogenous gases were partially re-assimilated, as seen by the increased total carbon recovered (~ 90%). This is in line with our previous observation of the carbon re-assimilation capability of \textit{C. beijerinckii} (Chapter 3). The missing 10% carbon may be the result of the stripping of solvents from the broth.

To our knowledge, this is the first report culturing \textit{C. beijerinckii} under aerobic conditions, suggesting a more robust oxidative stress defense mechanism than traditionally thought. Accordingly, in addition to the previously identified oxidative stress related genes in \textit{C. beijerinckii}, we identified additional ORFs (open reading frames) coding for putative NADH-dependent oxidoreductases. For example: flavo-diiron protein (Cbs\_4483), which reduces oxygen to water\(^{33}\); superoxide dismutase and reductase (Cbs\_1507, Cbs\_1856 and Cbs\_3348), that reduces superoxide anions\(^{28}\); and alkyl hydroperoxide / thiol peroxidase (Cbs\_0765, Cbs\_2959, Cbs\_2977 and Cbs\_3035), both responsible to \(\text{H}_2\text{O}_2\) reduction\(^{77}\).

Therefore, based on our physiological data and the putative oxidative stress related genes annotated in \textit{C. beijerinckii}, we propose a model that may explain the high oxygenase activity of this species, with or without MnKat, (Figure 2-5): (1) Oxygen is reduced to superoxide anion (\(\text{O}_2^-\)) and hydrogen peroxide [\(\text{H}_2\text{O}_2\)] by the interaction with flavoproteins. (2) Subsequently, \(\text{O}_2^-\) is converted to \(\text{O}_2\) and/or \(\text{H}_2\text{O}_2\) by a superoxide dismutase and superoxide reductase, respectively (along with spontaneous dismutation), and then (3) \(\text{H}_2\text{O}_2\) is reduced to water by rubrerythrin / rubredoxin peroxidases. (4) Additionally, oxygen is reduced to water by flavo-diiron proteins. These four reactions are NADH-dependent. Under aerobic conditions, dissolved oxygen is detected in the medium because the number of cells and the consequent oxygenase activity may become a bottleneck due to the excess \(\text{H}_2\text{O}_2\)
pool. Moreover, the cells deplete large amounts of redox resources to detoxify of $H_2O_2$, since its reduction to $\cdot HO$ through Fenton reactions are fatal$^{23,24}$. This is evident by the reduced growth of SA-1N compared to SA-1K under aerobic conditions. When cells contain an active MnKat (SA-1K), $H_2O_2$ is reduced to water and oxygen in a NADH-independent fashion, while saving reducing power and limiting the Fenton reactions$^{23,24}$. The extra NADH may be contributing to the oxygenase activity of the flavo-diiron proteins, since $O_2$ remains undetectable throughout the fermentation. As a result, as catalase removes the $H_2O_2$ pool, the reaction cascade is greatly improved, which is evident by the fitness increase of SA-1K. Additionally, the presence of an active laccase facilitates the reduction of oxygen to water, when a phenolic compound is present.

Overall, to our knowledge, this is the first report unveiling the aerotolerant phenotype of *C. beijerinckii*. This discovery, plus the laccase activity, advances our understanding of the physiology of this species, while inviting further investigations. Specifically, what is the activity of the different oxidative stress related genes annotated in *C. beijerinckii*? Moreover, while here we provide an efficient model for stable heterologous gene expression in *C. beijerinckii*, this is the first report of a heterologous manganese-catalase expression and activity in solventogenic *Clostridium*, and outside the LAB group.
2.6 Materials and Methods

2.6.1 Organisms

*Clostridium beijerinckii* SA-1 (ATCC 35702) is an offspring strain derived from NCIMB 8052. Literature about its genome and physiology are available\(^{56,78}\). This strain has...
been obtained from the American Type Culture Collection (ATCC). Its identity was verified by PCR amplification of an unique insertion sequence previously identified by our group\(^\text{56}\).

2.6.2 Bacterial medium and culture conditions

*C. beijerinckii* SA-1 stocks and inocula were prepared as consistently performed in our lab\(^\text{16,56}\). The growth experiments were performed in a medium previously designed by our group\(^\text{15}\) along with 1 mM MnCl\(_2\). We used 5 µg/mL erythromycin, (where 2 µg/mL is the minimum inhibitory concentration [MIC]) for inocula generation only. The base components of the medium were autoclaved and the sugar (6% w/v sucrose) and trace components were added aseptically to the medium reservoir by filtration (0.22 µm filters).

2.6.3 Chromosomal DNA isolation

Chromosomal DNA isolation was performed as previously described, with modifications\(^\text{79}\). Overnight-grown cultures were spun down and mixed with 200 µL lysis buffer (2% triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA pH 8.0). The tubes were frozen at -80°C for 5 min and thawed at 37°C for 5 min three times. Then the tubes were vortexed for 30 s and chloroform was added (200 µL). The tubes were then vortexed for 2 min and centrifuged at 20,000 x g for 3 min. The aqueous (top) layer was transferred to ice-cold 100% ethanol (400 µL) and was allowed to precipitate for 5 min. The tubes were then centrifuged for 5 min at 20,000 x g. After this, the liquid was removed and the sample washed with 500 µL 70 % ethanol. The tubes were centrifuged again as before and finally, the DNA was re-suspended in 50 µL molecular biology-grade water and stored at -20°C.
2.6.4 Vectors preparation and propagation

The Thl promoter in pMTL85151-PPS-FLP\textsuperscript{80} was first switched for the ferredoxin promoter (Originally from \textit{Clostridium pasteurianum}) found in pMTL007C-E2\textsuperscript{80}. To accomplish this, we PCR amplified the promoter using primers FDXproNotIF and FDXpro2NdeIR and ligated the fragment into pMTL85151-PPS-FLP NotI/NdeI sites yielding pMTL85151-FDXpro. The manganese-catalase gene and its promoter and terminator were PCR-amplified from \textit{Lactobacillus plantarum} CECT 221 using primers NdeIKatLpF2 and EcoRIKatLpR2 using the pfu polymerase (which produces blunt ended PCR fragments). This fragment was cloned into pBSKSII+ at the EcoRV site; yielding pBSKSII+Kat. The MnKat open reading frame was PCR amplified with KatNdeIATGF and KatNheIstopR and the Nde1-NheI digested fragment was ligated into pMTL85151-FDXpro Nde1-NheI sites to create pMTL85151-FDXpro-Kat. Lastly, a fragment comprising OriT/TraJ-FDXpro-Kat-FDXterm from pMTL85151-FDXpro-Kat, was amplified using primers OriTFBamHI and TermRXbaI, and cloned into pTRKH2 plasmid\textsuperscript{64} at BamHI and XbaI sites, yielding pTRKH2-OriT/TraJ-FDXpro-Kat-FDXterm. This erythromycin resistant plasmid contains \textit{E. coli} p15A and Gram-positive pAM\textbeta{}1 origins of replication s, respectively. Primers are listed in Table 2-3.

2.6.5 Transformation of \textit{C. beijerinckii} SA-1

The transformation of \textit{C. beijerinckii} was performed using electroporation as previously described, but with some modifications\textsuperscript{81}. The cells were activated as consistently performed in our lab\textsuperscript{56} and grown in 10 mL TYG medium (yeast extract 10g, glucose 20g, tryptone 30g and L-Cysteine 1 g, per L of water) at 37 °C on a bench-top incubator.
optical density was monitored on a digital spectrophotometer (SmartSpec Plus, BioRad, USA). Once an OD$_{600\text{nm}}$ of 0.78 was reached, the cells were harvested by centrifugation at 6000 rpm for 10 min at 4°C, and re-suspended in electroporation buffer (270 mM sucrose, 5 mM NaH$_2$PO$_4$, 1 mM MgCl$_2$, pH 6.4). The cells were washed with the same buffer two more times and resuspended to a final volume of 800 µL. The cells (400 µL) were then transferred to a 0.2 cm ice-cooled electroporation cuvette, and 1 µg plasmid DNA (10 µL) was layered on top of the cells without mixing and incubated on ice for 5 minutes. Electroporation was performed using a Gene Pulser Xcell (Bio-Rad, Hercules, CA) under the following conditions: 2.0 KV, 800 Ω and 25µF. After the pulse, the cells were immediately mixed with 400 µL warm (37°C) TYG. Subsequently, the cells were transferred into 9 mL fresh TYG medium, and incubated at 37°C in a Coy anaerobic chamber overnight for recovery. After observing intense gas production, the cells were centrifuged at 6000 rpm for 10 min at 4 °C and resuspended in 500 µL TYG medium. Next, the cells were spread on TYG agar plates containing 3 µg/mL erythromycin (Emr). The plates were incubated anaerobically at 37 °C for 24 h. Colonies resistant to erythromycin were selected and screened by colony-PCR to confirm the presence of the Emr and MnKat genes.

2.6.6 Preparation of dialyzed cell-free extracts (CFEs)

*C. beijerinckii* SA-1K and SA-1N (containing pTRKH2-KAT and pTRKH2, respectively) were grown in 250 mL Erlenmeyer flasks containing 70 mL M17 medium (Difco, Detroit, MI) with 5% (w/v) sucrose, 1 mM MnCl$_2$ and 10 µg/mL erythromycin, at 37°C. The growth was performed in a bench-top incubator under atmospheric air conditions. The method for preparing cell-free extracts was adapted from Givaudan *et al*[@]: The cells
were harvested at late-exponential growth phase (optical density at 600 nm \(\text{OD}_{600}\) of 1.6) and washed by centrifugation at 5000 \(x\) g for 10 min at 4 °C and resuspension three times in 0.01 M potassium phosphate buffer (pH 7.0). The final washing step was performed in 5 mL buffer and then disrupted by ultrasonic treatment (Vibracell, Bioblock) on ice, twice for 5 min with 15 s pulses. The cell debris was removed by centrifugation at 20,000 \(x\) g for 30 min at 4°C. The supernatant (cell-free extracts [CFEs]) were dialyzed four times at 4°C for 24 h, in 25 mM sodium phosphate buffer (pH 7.0). The CFEs were also concentrated using first a 100 and then a 10 KDa filter (EMD Millipore, Germany).
Table 2-3. Bacterial strains, plasmids and primers used in this study

<table>
<thead>
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<th>Strains</th>
<th>Features</th>
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<tr>
<td><em>E. coli</em> EC1000</td>
<td>RepA+ MC1000, KanR, carrying a single copy of the pWV01 repA gene in the glgB gene</td>
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<td><em>E. coli</em> ER2925</td>
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<td>[68]</td>
</tr>
<tr>
<td><em>C. beijerinckii</em> SA-1</td>
<td>Offspring of type-strain NCIMB 8052</td>
<td>ATCC</td>
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<tr>
<td><em>C. beijerinckii</em> SA-1K</td>
<td>+ pTRKH2-OriT/TraJ-FDXpro-Kat-FDXterm</td>
<td>This study</td>
</tr>
<tr>
<td><em>C. beijerinckii</em> SA-1N</td>
<td>+ pTRKH2</td>
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</tr>
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**Plasmids**

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<tr>
<td>pBSKSII+KAT</td>
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</tr>
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<td>pMTL85151-PPS-FLP3</td>
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<td>pMTL85151-OriT/TraJ-FDXpro-FDXterm</td>
<td>This study</td>
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<tr>
<td>pTRKH2</td>
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**Primers**

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<tr>
<td>SA-1 Insertion reverse</td>
<td>CTATCTCCTCTTCAATTCTATC</td>
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2.6.7 Biochemical assays

Dialyzed CFEs were analyzed for protein concentration by the Bradford method\textsuperscript{83}, using bovine albumin as the standard. Catalase activity was based on incubating the CFE with H\textsubscript{2}O\textsubscript{2} at 25 °C and measuring the disappearance of this compound in phosphate buffer at 240 nm\textsuperscript{84}. The laccase activity was performed as previously described with some modifications\textsuperscript{85}: CEFs were incubated with 250 µL 0.416 mM syringaldazine (Coefficient of Extinction ℇ = 65000 M\textsuperscript{-1}cm\textsuperscript{-1}) (Sigma-Aldrich, St. Louis, MO, USA), which is a canonical laccase substrate, along with 100 mM phosphate buffer (pH 6.0). The disappearance of the compound was measured at 595 nm and 37 °C. For this test, the buffer was saturated with air before use, as oxygen is co-substrate of laccase. 1 unit of laccase is 1 µmol of substrate (syringaldazine) oxidized into 1 µmol of product, per minute. Carbon-mol yields were calculated as previously reported\textsuperscript{86}.

2.6.8 Cultures of \textit{C. beijerinckii} SA-1 with air overlay

Growth experiments were performed in batch mode in a 2-Liter BIOSTAT B plus fermenter equipped with controllers for pH, temperature, agitation, and gas-mass flow controller (Sartorius BBI Systems, Germany). The temperature was set at 37°C, agitation speed at 250 rpm, and pH 6.5 by the automated addition of 0.5 N KOH and 25% (v/v) H\textsubscript{3}PO\textsubscript{4} into a working volume of 1,400 mL of culture. In order to maintain anaerobic conditions at inoculation, a continuous stream of sterile nitrogen gas (0.1 VVM) was sparged through the culture. Biomass proliferation in the fermentation tank was monitored using an in-line biomass sensor (Fundalux, Sartorius, BBI Systems, and Germany) and also by measuring the optical density (OD\textsubscript{600nm}) on a digital spectrophotometer (SmartSpec Plus,
BioRad, USA). After the cell density reached an OD$_{600nm}$ of 1, air was flowed through the headspace at 12.48 L/h. The dissolved oxygen (DO) was continuously monitored by an in-line INGOLD dissolved oxygen sensor (Mettler Toledo, OH, USA). Once dissolved oxygen reached 0% by sterile nitrogen sparging, air was flowed through steadily at 12.48 L/h. DO was measured in media without cells by sparging, overlay (headspace), and overlay with the agitation blades at the liquid/gas interphase (called here as disturbed overlay). The KLa was calculated as non-fermentative model (dynamic gassing-out) $\frac{dCL}{dt} = K_L a \left( C^* - CL \right)$. To evaluate the impact of endogenous fermentation gases on the culture, a continuous stream of sterile nitrogen gas (0.1 VVM) was sparged through the culture only at inoculation for 15 min. Subsequently, no gases were flowed through the reactor, during the fermentation. To maintain a constant 1 atm pressure within the reactor, a valve was connected to the exhaust, in order to release excess pressure. Sucrose was quantified with a high-performance liquid chromatography (HPLC) under isocratic conditions at 65°C, and a mobile phase of water at a 0.5 mL/min flow rate using a Supelcogel TM Ca column (300 mm x 7.8 mm, Supelco TM Analytical, Bellefonte, PA, USA) coupled to a refractive-index detector. The products of the fermentations (butanol, butyric acid, acetone, acetic acid and ethanol) were separated in a gas-chromatograph (GC) SS Porapak Q 80/100 column (OV, Marrietta, OH, USA) in a GC (GC-8A) fitted with a flame ionization detector (FID) (Shimadzu Corporation, Kyoto, Japan), using 200 kPa of nitrogen as the mobile phase with an injection temperature of 220°C and a column temperature of 140°C.
2.6.9 Cultures challenged with ferulic acid

_C. beijerinckii_ SA-1K (MnKat⁺) and SA-1N (MnKat⁻) were cultured at 37°C in defined medium¹⁵ containing 30 g/L sucrose as carbon and energy source. The initial pH was 6.50 and was not controlled throughout the experiments. The media contained 4 µg/mL erythromycin and increasing concentration of ferulic acid (0, 0.15, 0.3, 0.75, 1.0 and up to 1.2 g/L) (MP Biomedicals, Santa Ana, CA, USA). When required anaerobic conditions were maintained in a Coy anaerobic chamber, or aerobically in 50 mL conical tubes at 100 rpm, with 20 mL headspace.
2.7 Bibliography


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56. Sandoval-Espinola, W. J. *et al.* Comparative phenotypic analysis and genome sequence of *Clostridium beijerinckii* SA-1, an offspring of NCIMB 8052.


69. Bandyopadhyay, B. & Humphrey, A. Dynamic measurement of the volumetric oxygen


Chapter 3 - From greenhouse gases to biofuel: carbon capture to n-butanol by *Clostridium beijerinckii*

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**Under review**
3.1 Abstract

Recent efforts to combat increasing greenhouse gases emissions include their capture into advanced biofuels, such as butanol. Traditionally, butanol research has been centered solely on its generation from sugars. Our results show the mixotrophic CO$_2$ and H$_2$ reassimilation by n-butanol-producer *C. beijerinckii*. This was detected as synchronous CO$_2$/H$_2$ oscillations by direct (real-time) monitoring of the fermentation gasses. Additional functional analysis demonstrated syngas (H$_2$, CO$_2$ and CO) assimilation, increasing total carbon recovery above typical heterotrophic values. These results were further validated by genome- and transcriptome-wide analysis, revealing transcription of key Wood-Ljungdahl pathway and other autotrophy-related genes. Therefore, this report provides genetic and physiological evidence of inorganic carbon-capture by *C. beijerinckii*.

3.2 One Sentence Summary

*Clostridium beijerinckii* captures CO, CO$_2$ and H$_2$ into third-generation biofuels, n-butanol and butyrate.

3.3 Main text

Current societal efforts require solutions to address increasing greenhouse gas emissions$^1$. Accordingly, carbon-capture and its biotransformation into useful value-added commodities, including renewable biofuels, has become a major area of research. The Wood-Ljungdahl pathway of autotrophic organisms permits the assimilation of hydrogen and carbon dioxide (or carbon monoxide) into acetyl-CoA, which accumulates in the culture
broth as acetate or bioethanol\(^2\)-\(^6\). Currently, butanol is considered one of the ideal advanced renewable fuel due to a number of favorable properties and applications\(^7\)-\(^9\). For example, it can be used unblended in unmodified car engines and is compatible with current oil infrastructure\(^10\). However, only recently has the assimilation of syngas (H\(_2\), CO and CO\(_2\)) into butanol, by natural or genetically modified microbes, been assessed, and remains in the early stages of development\(^4\),\(^5\),\(^11\)-\(^13\). Therefore, in order to achieve cost-competitive butanol production, most research has focused on assessing the heterotrophic biotransformation of renewable feedstock by traditional solventogenic Clostridia. Interestingly, the reported data shows significant variability in final product yields, pointing towards overlooked pathways\(^8\),\(^14\)-\(^17\). With this in mind, we performed a deeper examination of the fermentation gases as physiological signals, while assessing the assimilation of syngas by the natural n-butanol producer \(C.\ beijerinckii\).

3.3.1 Real-time (inline) fermentation gas monitoring reveals CO\(_2\)/H\(_2\) oscillations

We performed a series of fed-batch fermentations of \(C.\ beijerinckii\) growing in balanced media\(^18\) with sucrose (6 \(\%\) [w/v]) as the carbon and energy source. We monitored in real-time (in-line) the endogenous gasses generated during the fermentation, while using a steady flow of nitrogen as a carrier (and to maintain anoxic conditions). We tested three feed compositions, all containing an additional 80 g of sucrose: (i) fresh complete medium; (ii) 2x trace components\(^18\), or (iii) sucrose only. Interestingly, direct measurements of H\(_2\) and CO\(_2\) generation showed in-phase synchronous oscillations coinciding with late log-phase and the onset of solventogenesis (when H\(_2\) and CO\(_2\) reached \(\approx 3 \%\) [v/v]) (Fig. 3-1). These types of fluctuations are normally observed in feedback-loop controls as a response to metabolic
pathway changes\textsuperscript{19,20}. Indeed, diauxic growth was evident from the decrease in the specific growth rate as \( \text{H}_2 \) and \( \text{CO}_2 \) resumed their accumulation (Fig. 3-S1). Since this behavior was not affected by the feed’s composition or its initiation time (i.e. before or after the oscillations), it suggested that neither organic carbon nor another medium component modulated this behavior. Regardless, the cells failed to utilize all the provided sugar, and its utilization varied significantly among experiments (Fig. 3-S2, D, D1). In contrast, the kinetic and yield parameters for products and biomass did not vary significantly (Fig. 3-S2 - A1, B1, and C1; and Table 3-S1). Interestingly, it was previously shown that recirculating endogenous \( \text{H}_2 \) and \( \text{CO}_2 \) during butanol fermentation (for maintaining anoxic conditions) allows for more sugar consumption and acid generation\textsuperscript{14}. In bacteria that harbor the Wood-Ljungdahl pathway, the hydrogen-dependent \( \text{CO}_2 \) reduction is also a mechanism for redox balance, helping to sustain substrate uptake\textsuperscript{21}. Nevertheless, this pathway has not been previously described in any of the traditional solventogenic \textit{Clostridium} species\textsuperscript{5,6}. 
Figure 3-1. Direct monitoring of hydrogen and carbon dioxide evolution in the gas-phase during fed-batch fermentations by *C. beijerinckii*. H₂ and CO₂ evolution from three independent experiments performed in a Biostat B+ reactors using defined medium containing 6% (w/v) sucrose as limiting carbon and energy source with an initial and final volumes of 1000 mL and 1400 mL, respectively. Feeds (400 mL) contained 80 g sucrose fed at 0.08 mL/h, to reach a final concentration of 100 g/L (w/v) along with: Red line: only sugar was added; Black line: fresh whole medium and; Blue line: 2X trace components. Yellow boxes show detail of the H₂ and CO₂ oscillation. Fermentations were controlled at 250 rpm, 37 °C and pH 6.5 and constant sparging (12.48 L/h) of nitrogen gas was achieved using mass flow controllers. Output gas-phase composition was continuously monitored and recorded using two analyzers: An EasyLine continuous analyzer, model EL3020 (ABB, Germany) and a Pfeiffer OmniStar quadrupole mass spectrometer.

3.3.2 Genomic and transcriptomic analysis indicate expression of key Wood-Ljungdahl pathway genes

To explain the observed oscillations, we performed an *in silico* search for the polyphyletic Wood-Ljungdahl pathway genes within the *C. beijerinckii* genome. We found open reading frames that putatively code for acetyl-CoA synthase / CO dehydrogenase (Cbei_5054-56/Cbei_3020), formate dehydrogenase (Cbei_3801, Cbei_3794 and Cbei_3798), formyl-THF ligase (Cbei_0101), methylene-THF dehydrogenase / cyclohydrolase (Cbei_1702) and methylene-THF reductase (Cbei_1828). The putative
proteins encoded by these genes have high sequence identity to those of *Clostridium ljungdahlii*, the species most often utilized for ethanol generation from syngas (Fig. 3-2 A)\(^4,12\). The key enzymes, acetyl-CoA synthase / CO dehydrogenase and formate dehydrogenase, have 77.62 and 72.23% sequence identity, respectively. The former is the main enzyme within the carbonyl branch of the Wood-Ljungdahl pathway, and the final step of the methyl branch (or initial step, if CO is supplied). The latter initiates the methyl branch, allowing CO\(_2\) capture. Both paths lead to the generation of acetyl-CoA. Additionally, *C. beijerinckii* contains Fe-only and NiFe-hydrogenases (Cbei\_1773, Cbei\_3796, Cbei\_4110 and Cbei\_3013) with similarities to those of *C. ljungdahlii*; in this species, along with H\(_2\) generation, these enzymes allow for hydrogen uptake capabilities, providing extra reducing equivalents to the pathway\(^4\).

Focusing on these genes, we performed an analysis of publicly available transcriptomic data from batch cultures of *C. beijerinckii*\(^23\). An RNA-seq time-course experiment was previously reported by Wang *et al*\(^24\) using cells growing in P2 medium sparged with pure nitrogen. After quality trimming and normalization for gene length and number of assembled reads, we found all of the putative Wood-Ljungdahl pathway genes to be expressed, either constitutively (Cbei\_5056 and Cbei\_1828) or differentially over time (Cbei\_1702, Cbei\_0101, Cbei\_3801, Cbei\_3794, Cbei\_3798 Cbei\_3020 and Cbei\_5054) (Fig. S3). We identified expression changes coinciding with the CO\(_2\)/H\(_2\) oscillations, pointing towards the re-assimilation of these gases (Fig. 3-1 and Fig. 3-2 B). Among these genes, formate dehydrogenase showed the lowest expression level in the evaluated experimental condition (i.e. low biomass and CO\(_2\)/H\(_2\) concentration, and nitrogen atmosphere); however, increasing towards mid-log-phase, concurring with the time-point where CO\(_2\) and H\(_2\)
accumulation is maximal (Fig. 3-2 B). Among the four hydrogenase genes annotated, two displayed the highest expression levels: the correlation between expression and hydrogen oscillation/evolution suggested that Cbei_3013 and Cbei_1773 are primarily used for H₂ uptake and H₂ evolution, respectively. This analysis provided indirect evidence that, in C. beijerinckii, these gases may regulate the Wood-Ljungdahl pathway expression. Similar inducible behavior was observed in cultures of acetogens Clostridium thermoautotrophicum and C. ljungdahlii. Additionally, the genes encoding a carbonic anhydrase also showed expression (Cbei_4425/Cbei_1031). This enzyme allows for even more intracellular CO₂ availability. This transcriptional data suggests that under our culture conditions (higher biomass and CO₂/H₂ levels), C. beijerinckii may possess an active Wood-Ljungdahl pathway.
Figure 3-2. Wood-Ljungdahl (WL) scheme pathway in *C. beijerinckii* and time-course expression profile of corresponding genes. A) WL scheme pathway in *C. beijerinckii* and associated locus tags. Black squares represent presence of putative genes within *C. beijerinckii* genome. Boxes in different gray intensities represent the *Clostridium ljungdahlii* annotated genes and the comparative percentage identities values (39), with darker shades indicating higher identity. Salmon arrows indicate reactions predicted to be catalyzed by Acetyl-CoA synthase/CO dehydrogenase. B) Time-course expression profiles of WL pathway predicted genes, along with genes related to energy conservation and carbon fixation, in *C. beijerinckii*. The FPKM (fragments per kilobase per million) were calculated from publically available RNA-seq data (24). Lines represent CO$_2$ and H$_2$ evolution in the gas-phase of *C. beijerinckii* growing in defined medium (18), 37 °C, 250 rpm, and constantly sparged with pure nitrogen (12.48 L/h).
3.3.3 Functional evaluation shows inorganic carbon capture by *C. beijerinckii*

In order to confirm the above-described indirect evidence, we performed mixotrophic (sucrose 3% and fructose 1.5% [w/v]) chemostat fermentations ($D = 0.135 \text{ h}^{-1}$) while steadily sparging CO$_2$ and H$_2$ at high and low concentrations, balanced with nitrogen. We observed steady-state consumption of CO$_2$ and H$_2$ along with proportional increases of product yields values above theoretical levels (Fig. 3-S4). If sucrose and fructose were the only carbon and energy sources, yields should have remained at or below the theoretical maximum (i.e. 0.66 % C-mol, considering one decarboxylation from 3C pyruvate to 2C Acetyl-CoA). The higher-than-maximum yields indicated additional carbon assimilation was only possible by inorganic carbon capture.

Considering current efforts to transform surplus syngas into biofuels$^{5,13}$, we also sparged this gas at increasing step-wise concentrations (Table 3-S2). Specifically, we sparged syngas mixtures from low (9%), to medium (32%), to high (60%) concentrations, balanced with nitrogen (100% syngas contained 20% CO, 20% CO$_2$, 10% H$_2$ and 50% N$_2$). At low syngas concentration, *C. beijerinckii* was able to oxidize CO, releasing H$_2$ and CO$_2$ as shown by the steady-state values (Fig. 3-3 A). Accordingly, there was a nonsignificant difference in C-mol yield and carbon-energy recovery balance compared to the control. Additionally, the steady-state values of sugar utilization were improved, resulting in higher product titers (Fig. 3 B). Similar behavior has been observed in acetogens *Clostridium thermoaceticum*, *C. autoethanogenum*, *Rhodopseudomonas gelatinosa* and also *Carboxydothermus hydrogenoformans*, according to the following reaction: CO + H$_2$O $\rightarrow$ CO$_2$ + H$_2$, mainly used for redox balance when grown mixotrophically$^{11,21,28}$. The Gibbs free energy of the reactions involving the acetyl-CoA synthase / CO dehydrogenase and formate dehydrogenase favors
conversion of CO to acetyl-CoA and to CO₂ and H₂, rather than CO₂ to CO or to formate, respectively. This futile cycle in *C. beijerinckii* under low syngas exposures, may indicate that the acetyl-CoA synthase / CO dehydrogenase has higher affinity for CO than CO₂. Thus, the whole pathway does not proceed to acetyl-CoA (i.e. carbon assimilation) because CO₂ is not reduced to formate, rendering the methyl branch activity incomplete under this condition.

As also observed in the transcriptomic data (i.e. higher expression when CO₂/H₂ were maximal), this physiological behavior suggests that CO₂ is the limiting component of the Wood-Ljungdahl pathway in *C. beijerinckii*. Accordingly, when cultures were exposed to higher syngas concentrations, higher-than-theoretical C-mol yields and carbon/energy recovery mass balances were detected with the concomitant consumption of H₂, CO, and CO₂, (Fig 3-3 A, C, D). Specifically, at medium and high syngas concentrations, 11 and 17% more carbon, and 19 and 27 % more carbon and electrons, respectively, were recovered. Interestingly, butanol and butyric acid increased by 5.5- and 1.85-fold, respectively, while biomass did not change significantly, which is typical for this catabolic pathway. Although butanol is the main target in ABE fermentation, butyric acid is also a value-added product and can be reassimilated into *n*-butanol through multi-stage fermentations. Additionally, the proportion of total carbon in the form of *n*-butanol, increased by 92%. The generation of C-4 compounds, such as butyric acid and butanol, require more NADH than C-2 compounds (such as ethanol), underscoring the cells emphasis in recycling electrons.

The gas assimilation rate, larger than the saturation values in each condition, also indicated biological activity (Fig. 3-3 A and Fig. 3-S5). We also tested higher syngas concentrations (up to 100% syngas containing 20% CO, 20% CO₂, 10% H₂ and 50% N₂), showing carbon capture but generating lower yields, probably due to carbon monoxide
poisoning\textsuperscript{31} (Fig. 3-S6). This indicates that the working window for mixotrophic syngas fermentation by \textit{C. beijerinckii} is between 30 and 60\% syngas. This is lower than the working conditions utilized in cultures of \textit{C. ljungdahlii} and \textit{C. carboxidivorans}, also suggesting that the carbon-capture enzymatic affinity of \textit{C. beijerinckii} may be higher\textsuperscript{12,13}. In addition to improved yields, with increasing electron sink availability, more fructose was consumed leading to higher final product titers (Fig. 3-3 B).

We have also performed batch fermentations of \textit{C. beijerinckii} under a continuous flow of high syngas concentration, as sole carbon and energy source. We observed transient cell proliferation and CO assimilation (not shown). However, cell growth and gas assimilation stopped in early exponential growth phase, as the cells initiated sporulation. As a result, no products were detected. Considering that we control pH, nutrient limitation\textsuperscript{32}, probably in the form of organic carbon, was the trigger for sporulation for \textit{C. beijerinckii}.
Figure 3-3. Kinetic and yield parameters of syngas (CO, CO$_2$ and H$_2$), substrate utilization, product generation, and carbon and energy balances in steady-state at D = 0.135 h$^{-1}$. (A) Each tested gas composition was constantly sparged at 12.48 L/h. Steady-state values of gas generation (H$_2$ and CO$_2$) and syngas utilization were obtained after subtracting the net values of H$_2$ and CO$_2$ generated under sparged nitrogen from the values of output gas phase for each condition. Zero value indicates input = output. Negative values indicate more gases being produced than input. Positive values indicate the steady-state amounts continuously assimilated. (B) Sucrose and fructose consumed in steady states under the different tested gas phase condition. (C) Yield (C-mol product/C-mol carbon source utilized) and (D) carbon and energy balance, calculated as $\frac{Y_{p} \gamma}{Y_{x} \gamma} - 1$, where Y$_p$ and Y$_x$ represent C-mol ratios on figure (C); $\gamma$ represents electrons available for each fermentation product (40). Syngas mixture was balanced with nitrogen (100% syngas contains 20% CO, 20% CO$_2$, 10% H$_2$ and 50% N$_2$). The results presented here were obtained from three biological replicates and the represented means are values at steady-state conditions from at least three samples extracted at different retention time intervals. Significance at 0.05 refers to comparisons between whole columns.
3.3.4 Nitrite as an electron sink for energy conservation

Under mixotrophic growth, the Wood-Ljungdahl pathway operates mainly for electron recycling\textsuperscript{21,29}. Consequently, another way to demonstrate the presence of an active pathway is to inhibit CO assimilation by providing an alternative and preferred electron acceptor. Both nitrate and nitrite are known to have this effect on CO assimilation by acetogenic bacteria\textsuperscript{33,34}. In order to test this hypothesis in \textit{C. beijerinckii}, we performed chemostat pulse experiments under high syngas concentration (i.e. 60\% [v/v]). Interestingly, nitrate showed no effect on \textit{C. beijerinckii}. However, less-reduced nitrite partially inhibited CO assimilation (1 mol per mol of NO\textsubscript{2}), while increasing hydrogen consumption (2.5 mol of H\textsubscript{2} per mol of NO\textsubscript{2}). Additionally, biomass increased proportionately, shifting the pathway from catabolism to anabolism (Fig. 3-4). Both electron acceptors have also been shown to increase biomass in acetogens \textit{C. thermoautotrophicum} and \textit{Moorella thermoacetica}\textsuperscript{33,34}. The H\textsubscript{2}-dependent CO\textsubscript{2}, or CO assimilations are thermodynamically unfavorable as they do not generate a gain in ATP\textsuperscript{29}. Thus, nitrite reduction is preferred as a less expensive way to recycle electrons. As such, the nitrite reductase reaction requires only electrons, in the form of hydrogen and reduced ferredoxin (i.e. not ATP). \textit{C. beijerinckii} contains a putative ferredoxin-nitrite reductase (Cbei_0832), likely responsible for the observed phenotype, which also unveils this species as a facultative nitrite dissimilator.
Figure 3-4. Transient responses to nitrite pulses by C. beijerinckii growing in chemostat (D = 0.135 h⁻¹). Experiments were performed on defined medium (18) containing 3 % sucrose and 1.5 % fructose (w/v) and sparged with 60% (v/v) syngas balanced with nitrogen (100% syngas contains 20% CO, 20% CO₂, 10% H₂ and 50% N₂) at 37 °C. The additions of sodium nitrite are indicated with vertical dashed lines to reach final concentrations as follow; 3.1 mM (A), 6.2 mM (B) and 12.4 mM (C). The CO and H₂ data shown were obtained by monitoring, in real time, with an EasyLine continuous gas analyzer, model EL3020 (ABB, Germany). Nitrite concentrations higher than 24 mM proved toxic and led to washout. Steady-state values were re-established prior to testing each nitrite concentration. Correlations of NO₂ added with D) H₂ consumed, E) amount of CO consumption displaced, and F) biomass increase, were calculated from the slopes after fitting the data to linear regressions.

3.3.5 Transcription of autotrophic energy-conservation genes

Considering the poor energetics of the Wood-Ljungdahl pathway, acetogens rely either on substrate-level phosphorylation or on chemiosmosis for ATP synthesis.⁶,²¹,²⁹ Chemiosmotic, or ion gradient-driven phosphorylation, is the only energy conservation mechanism during autotrophic growth. In acetogens lacking cytochromes, Na⁺ is required. Otherwise, H⁺ is coupled to a motive electron transport chain. In both cases, the ion gradient allows for energy generation through ATP-synthases. B-type cytochromes are responsible for H⁺-dependent ATP generation, and can be coupled to a membrane-bound methylene-THF reductase.³⁵ A third type has been described in C. ljungdahlii, which relies on a Rnf-complex
but not cytochromes\textsuperscript{4,36,37}. Interestingly, the \textit{C. beijerinckii} genome encodes cytochromes (also involved in nitrite reduction\textsuperscript{38}) \textit{b}-type (Cbei\_2439), \textit{c550} (Cbei\_2762), \textit{c551} (Cbei\_4151), \textit{c} biogenesis protein (Cbei\_2976), cytochrome-bound flavoproteins (Cbei\_3109), and also genes coding for the Rnf-complex (Cbei\_2449-2454). Additionally, the methylene-THF reductase of \textit{C. beijerinckii} is predicted\textsuperscript{39} to contain transmembrane domains. Our transcriptomic analysis showed high expression levels of all these energy-conserving genes in \textit{C. beijerinckii}, especially the Rnf-complex (Fig. 2). Ironically, sporulation triggered by organic carbon limitation does not allow \textit{C. beijerinckii} to growth autotrophically. This indicates that these chemiosmotic mechanisms are useful only in mixotrophic cultures of \textit{C. beijerinckii}. Interestingly, \textit{C. ljungdahlii} also requires the Rnf-complex when cultured mixotrophically\textsuperscript{37}.

### 3.3 Discussion

We have shown that, under mixotrophic conditions, \textit{C. beijerinckii} captures inorganic carbon and electrons, increasing product yields above theoretical heterotrophic values. Therefore, we propose a logic model that explains the carbon-electron flow of a mixotrophic culture of \textit{C. beijerinckii} (Fig. 3-5). In the presence of CO and CO\textsubscript{2}, there are three possible paths for carbon capture: 1) CO\textsubscript{2} to carbonate through carbonic anhydrase, or 2) CO oxidation to generate CO\textsubscript{2} + H\textsubscript{2}, if the CO\textsubscript{2} in the gas-phase is < 5 \% (v/v); or finally, 3) the complete Wood-Ljungdahl pathway, if CO\textsubscript{2} > 5\%. Simultaneously, supplied sugars proceed to glycolysis. In the absence of an electron bottleneck, ABE-fermentation utilizes all the sugar-derived carbon and electrons. Otherwise, and if no external electron sink is provided, fermentation stops, limiting sugar utilization. In the presence of an external electron sink
(such as CO/CO₂), #2 or #3 take place. If #3 takes place, the extra acetyl-CoA generated, along with the still-running ABE-pathway, leads to 17 and 27 % more carbon and carbon-energy recovered, respectively.

This discovery of inorganic carbon-capture by mixotrophic cultures of *C. beijerinckii* has important implications for our understanding of the physiology of this industrial butanol-producer, and adds a new biological alternative for greenhouse gas-capture. The product yield variability reported in the literature and the empirical records of microbial solvent production demonstrated the need for a deeper study of the biological gas-phase as signals for overlooked pathways. Our results show that *C. beijerinckii* captures inorganic carbon through the Wood-Ljungdahl pathway, increasing carbon and energy recovery above theoretical levels. Moreover, this physiological capability improves the product titers by increasing sugar utilization. Furthermore, the synchronous H₂/CO₂ oscillation is an example of a natural integrated oscillator, that can potentially be used for feedback controls such as biosensors⁴⁰,⁴¹.
Figure 3-5. Logic model of carbon-electron flow in *C. beijerinckii* grown mixotrophically. The data suggest that in the presence of CO and COR₂R, there are three possible paths for carbon capture: 1) COR₂R to carbonate through carbonic anhydrase, or 2) CO oxidation to generate COR₂R + H, if the COR₂R in the gas-phase is < 5 % (v/v); or finally, 3) the complete Wood-Ljungdahl pathway, if COR₂R > 5%. Simultaneously, supplied sugars proceed to glycolysis. In the absence of an electron bottleneck, ABE-fermentation utilizes all the sugar-derived carbon and electrons. Otherwise, and if no external electron sink is provided, fermentation stops. In the presence of an external electron sink (such as CO/COR₂R), #2 or #3 take place. If #3 takes place, the extra acetyl-CoA generated, along with the still-running ABE-pathway, leads to up to 17 and 27 % more carbon and carbon-energy recovered, respectively.
3.4 Acknowledgements

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3.5 Bibliography


3.6 Materials and Methods

3.6.1 Organisms

Clostridium beijerinckii SA-1 (ATCC 35702) was obtained from the American Type Culture Collection (ATCC). Its identity was verified by PCR amplification and sequencing of the 16S rRNA gene using the prokaryotic 16S rDNA universal primers 515F (5-
3.6.2 Bacterial medium and inocula preparation

*C. beijerinckii* stocks were activated as previously described and were grown in a previously designed medium. The base components were autoclaved and the sugar (6% w/v sucrose) and trace components were added aseptically to the medium reservoir by filtration (0.22 µm). The inocula were prepared as consistently performed by our lab. Exact fermentation conditions are detailed in the Main Text section.

3.6.3 Bacterial culture conditions

Growth experiments were performed in fed-batch or chemostat modes of operation in a 2-Liter Biostat Bplus fermenter equipped with controllers for pH, temperature, agitation, and gas mass-flow (Sartorius BBI Systems, Germany). The temperature was set at 37°C, agitation speed at 250 rpm, and pH 6.5 by the automated addition of 0.5 N KOH or 25% (v/v) H₃PO₄, into a working final volume of 1,400 mL of culture for fed-batch or 700 mL for chemostat. The fed-batch fermentations were started with 6 % (w/v) sucrose, and 400 mL containing 80 g of the same sugar were added at constant feed rate (0.08 mL/h) to reach a final concentration of 100 g/L (w/v). The initial volume was 1 L and final 1.4 L. Exact feed components and time of feed start are detailed in the Main Text section. For the chemostat experiments the conditions were identical as described for fed-batch except the carbon and energy source were 3% (w/v) sucrose and 1.5% fructose. Once the cells reached exponential phase under sparged pure nitrogen (OD600nm ~ 1), the feed and harvest flow were initiated.
and adjusted to a dilution rate $D = 0.135 \, \text{h}^{-1}$. Exact sparged gas compositions are detailed in the Main Text section, steady-state conditions were verified for each condition and at least three retention times were allowed before sampling was initiated. Three samples at each steady-state condition were obtained from at least one retention time intervals. The discrete ratios of continuous gas streams were always sparged at 12.48 L/h. Different gas-phase conditions, from pure nitrogen gas to increased syngas concentrations, were achieved by modifying the mix ratios between syngas and nitrogen using two mass flow controllers; the exact concentrations tested are detailed in the Results section (Table S2). Inlet and exhaust gases in the gas-phase ($O_2$, $N_2$, $CO$, $CO_2$, $H_2$, and $Ar$) were monitored and recorded in real-time using in-line $O_2/CO_2$ and $H_2/CO$ EasyLine continuous gas analyzers, model EL3020 (ABB, Germany), and a Pfeiffer OmniStar quadrupole mass spectrometer. Biomass proliferation in the fermentation tank was monitored and recorded using an in-line biomass sensor (Fundalux, Sartorius, BBI Systems, and Germany) and also by discrete measurements of the optical density ($OD_{600\text{nm}}$) on a digital spectrophotometer (SmartSpec Plus, BioRad, USA). Dry weight concentration was obtained by filtering a portion of sample using vacuum suction through a 0.2-µm-pore-size filter of known mass (mixed cellulose esters; EMD Millipore, Germany); the filter was then dried at 60 to 70°C for 7 days and reweighed until constant weight.

### 3.6.4 Sample analysis

Sucrose, fructose, acetic and butyric acid were quantified with a high-performance liquid chromatograph (HPLC) under isocratic conditions at 65°C, and a mobile phase of water at a 0.5 mL/min flow rate using a Supelcogel TM Ca column (300 mm x 7.8 mm, Supelco TM Analytical, Bellefonte, PA, USA) coupled to a refractive-index detector.
Solvents (acetone, butanol and ethanol) were separated in a gas-chromatograph (GC) SS Porapak Q 80/100 column (OV, Marrietta, OH, USA) in a GC (GC-8A) fitted with a flame ionization detector (FID) (Shimadzu Corporation, Kyoto, Japan), using 200 kPa of nitrogen as the mobile phase with an injection temperature of 220°C and a column temperature of 140°C.

3.6.5 Proteins sequence identity analysis

Protein sequence identity were performed as previously described.

3.6.6 RNA-seq analysis

The sequence reads from the transcriptional profiling experiment of Wang et al\textsuperscript{24} were downloaded from the NCBI Sequence Read Archive (SRA accession number SRA045799) and imported into the Cyverse Collaborative Discovery Environment\textsuperscript{42}. The sequence reads were quality filtered with the trimmomatic program\textsuperscript{43} using the trimmers, “LEADING:5 TRAILING:5 SLIDINGWINDOW:4:15 MINLEN:36”. Two independent platforms were subsequently used to analyze these normalized data, Cyverse Discovery Environment and Geneious v9 (Biomatters Ltd., New Zealand), while aligning the sequences to the \textit{C. beijerinckii} NCIMB8052 genome (GenBank accession CP000721.1). In Cyverse, the sequences were aligned with tophat\textsuperscript{44} using the default parameters, while differences in transcript abundance were determined using the Cuffdiff program, which is part of the Cufflinks software package\textsuperscript{45}. The analysis in Geneious were performed using default parameters.
3.6.7 Gas calculations

For gas solubility we used Henry’s law: $C = k \times p$ where: $C$ is concentration, $k$ is Henry’s constant at 37 °C and $p$ is partial pressure. The $k$ values used were (in g/L): 0.0225 for CO; 1 for CO$_2$; 0.033 for O$_2$ and 0.0014 for H$_2$. To calculate gas consumption: $C = (O - I + E) \times (-1)$, where $O$ is output, $I$ is input, and $E$ is the amount the cells endogenously generate under nitrogen. Positive values indicate consumption. Negative values indicate generation.

3.6.8 Stoichiometry

For this calculation we used the methods previously reported$^{48}$.

3.6.9 Nitrite pulse experiments

We performed continuous culture pulse experiments with different concentrations of nitrite in the form of sodium nitrite (Sigma-Aldrich Inc., Saint Louis, MO, USA). Exact conditions are detailed in the Main Text section.
3.7 Supplemental Materials

Fig. 3-S1

Figure 3-S1. Mean growth curve of multiple fed-batch fermentations of *C. beijerinckii*. Two slopes, at early exponential (m_a) and late exponential (m_b) growth phases, respectively, are shown. $\mu_a$ and $\mu_b$ are early and late specific growth rates, respectively.
Figure 3-S2. Product and substrate profiles of fed-batch fermentations of *Clostridium beijerinckii*. Different feed nutritional compositions were used: Circle: whole medium + sucrose; Triangle: sucrose only and Square: 2x trace components + sucrose. Experiments were performed in a defined medium (Heluane et al, 2011) containing an initial amount of 6% (w/v) sucrose as limiting carbon and energy source. The final sucrose concentration (feed + initial medium) was 100 g/L, fed at 0.08 mL/h. Temperature was 37 °C and pH was controlled (6.5). initial and final volumes were 1 and 1.4 L, respectively. Nitrogen gas was flowed filtered-sterilized at 12.48 l/h throughout each experiment. A: Butanol; B: Acetone; C: Ethanol and; D: residual sucrose. A1, B1, C1 and D1 show the mean values and 95% confidence limits of butanol, acetone, ethanol and sugar, respectively. The vertical lines in D represent the time at which each feed was started: solid: whole medium + sucrose; dashed: sugar only and; dots: 2x trace components + sucrose. The solid vertical line in D1 represents the mean time at which feed was started. Error bars indicate SD.
Figure 3-S3. Time-point genome-wide expression profile and comparison of C. beijerinckii NCIMB 8052. A) Time-point genome-wide expression levels ($\log_2$ of fragment per kilo base per million -FPKM-), minus housekeeping genes (HKG) and those whose expression were below $\log_2 = 2$, in C. beijerinckii NCIMB 8052, highlighting Wood-Ljungdahl (WL) pathway genes. B) Volcano plots comparing expression levels of each time point. Alpha: 0.1; fold change considered: 1 $\log_2$ fold.
Figure 3-S4. CO$_2$ and H$_2$ consumption, carbon recovery and carbon and energy balance. (A) Steady-state values ($D = 0.135$ h$^{-1}$) of CO$_2$ and H$_2$ utilization calculated as absolute values of amount of H$_2$ and CO$_2$ produced by the cells under nitrogen conditions plus exogenous gases minus output. Positive values indicate the amount that the cells continuously assimilate, at a flow of 12.48 L/h. (B) Yield C-mol ratio and (C) carbon and energy balance, calculated as $\frac{Y_p}{Y_x} \times \gamma$, where $Y_p$ and $Y_x$ represent C-mol ratios of each product and biomass; $\gamma$ represents electrons available(40). The results presented here were obtained from three biological replicates and the represented means are values at steady-state conditions from at least three samples extracted at different retention time intervals. Significance at 0.05 refers to comparisons between whole columns.
Fig. 3-S5. Estimation of dissolved gases in the medium. Saturation values of each gas in the liquid phase, calculated according to Henry’s laws, for gas and liquid at 37 °C at different partial pressures.
Fig. 3-S6

Fig. 3-S6. Syngas (100%) consumption, carbon recovery and carbon and energy balance. (A) Steady-state values (D = 0.135 h⁻¹) of syngas utilization (CO, CO₂ and H₂) calculated as absolute values of amount of H₂ and CO₂ produced by the cells under nitrogen conditions plus exogenous gases minus output. Flow = 12.48 L/h. (B) Yield C-mol ratio and (C) carbon and energy balance, calculated as $\frac{Y_p}{\gamma}$, where Yp and Yx represent C-mol ratios of each product and biomass; γ represent electrons available[40]. The results presented here were obtained from three biological replicates and the represented means are values at steady-state conditions from at least three samples extracted at different retention time intervals. Significance at 0.05 refers to comparisons between whole columns.
### Table 3-S1

**Table 3-S1. Final kinetic and yield parameters.** Values were obtained after different fed-batch fermentations of *Clostridium beijerinckii* at 37 °C, pH 6.5 and feed at 0.08 mL/h, in defined media.

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<th>Variables and Parameters</th>
<th>Values</th>
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</thead>
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</tr>
<tr>
<td></td>
<td>Biomass yield (OD$_{600}$/g)</td>
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<tr>
<td></td>
<td>Butanol (g/L)</td>
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<tr>
<td></td>
<td>Acetone (g/L)</td>
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<tr>
<td></td>
<td>Ethanol (g/L)</td>
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Table 3-S2

Table 3-S2. **Experimental percentage of measured input syngas.** Inputs were balanced with N\textsubscript{2}, and corresponding values of its components as percentage of volume and millimols per hour. Flow: 12.48 L/h. Syngas 100% contains 20% CO, 20% CO\textsubscript{2} and 10% H\textsubscript{2}.

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<td>0</td>
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<tr>
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<td>11.17</td>
<td>54.77</td>
<td>11.01</td>
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</tbody>
</table>
Appendices
Appendix 1 – Correlation between sucrose utilization by *Clostridium beijerinckii* and ammonium availability, and effect of tungsten on ABE final titers and sucrose consumption.

Figure 4-1. Correlation between sucrose utilization by *Clostridium beijerinckii* and ammonium availability, and effect of tungsten on ABE final titers and sucrose consumption. (A) Correlation between sucrose consumed and ammonium availability in batch fermentations of *C. beijerinckii* SA-1. Defined media, containing 2X trace components (Heluane, 2011), along with 6 % (w/v) sucrose as limiting carbon and energy source. Temp: 37 °C, Vol: 100 mL in 250 mL bottles, initial pH 6.5 in anaerobic conditions. Overnight cultures were added pulses containing 4% sucrose (w/v) along with different concentrations of either ammonium acetate or glutamine, normalizing for NH₃⁺ content. (B) Effect of tungsten (Na₂WO₄) on acetone-butanol-ethanol (ABE) final titer and sucrose utilization by *C. beijerinckii* SA-1. Batch fermentations were performed in defined media (Heluane, 2011) containing 6 % (w/v) sucrose as limiting carbon and energy source. Temp: 37 °C, Vol: 100 mL in 250 mL bottles, initial pH 6.5. Experiments performed in anaerobic conditions.
Appendix 2 - Endogenous fermentation gases by *Clostridium beijerinckii* SA-1.

**Figure 4-2.** Endogenous fermentation gases by *Clostridium beijerinckii* SA-1. (A): CO$_2$ and H$_2$ generated during batch fermentation of *C. beijerinckii* SA-1, without gas stripping. Defined media (Heluane, 2011), containing sucrose (6% v/v) as limiting carbon and energy source. Temp: 37°C, pH 6.5, 250 rpm. (B): CO$_2$ and H$_2$ yields (mol of each gas per C-mol of substrate) in chemostat (D: 0.135 h$^{-1}$), fed-batch (initial 60 g/L and final 100 g/L sucrose, fed at 0.08 mL/h); and batch (60 g/L sucrose), in defined media, pH: 6.5, temp: 37°C, 250 rpm, with N$_2$ stripping (i.e. no CO$_2$ or H$_2$ assimilation) Inlet and exhaust gases in the gas-phase (O$_2$, N$_2$, CO, CO$_2$ and H$_2$) were monitored and recorded in real-time using in-line O$_2$/CO$_2$ and H$_2$/CO EasyLine continuous gas analyzers, model EL3020 (ABB, Germany), and a Pfeiffer OmniStar quadrupole mass spectrometer.