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# Investigation of growth modes of the lithoautotrophic bacterium *Cupriavidus necator*

Master's Degree Project in Applied Microbiology

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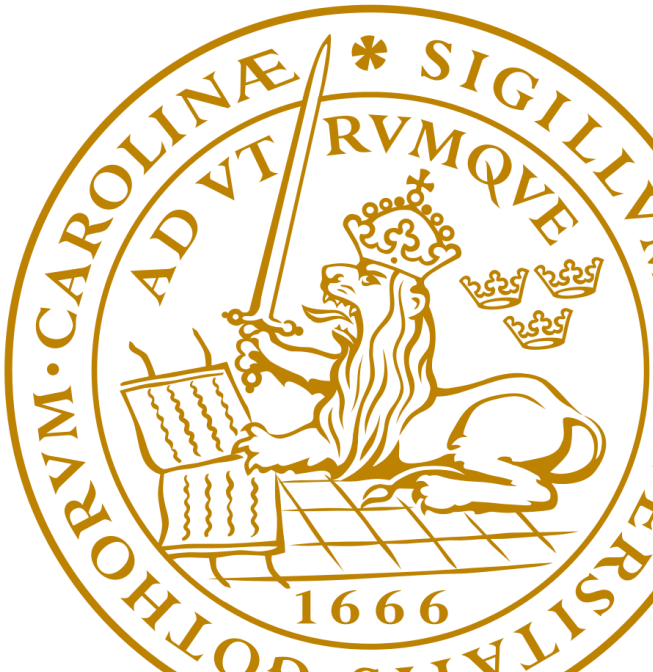

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# Abstract

The microorganism *Cupriavidus necator* is a very interesting bacterium that has been established as a model microorganism for its capability to produce polyhydroxyalkanoates, more specifically polyhydroxybutyrate (PHB), a potential precursor/raw material for bioplastics. Lately, the scientific community has raised interest in the chemolithoautotrophic nature of *C. necator* as it can be grown using $CO_2$ and $H_2$ as its only carbon and energy sources, respectively. Alternatively, it can also be grown under organoautotrophic conditions, where formic acid is used as substrate that is further metabolized in the cell to obtain $CO_2$ and reduced NADPH. The organoautotrophic growth mode is presumably similar to lithoautotrophic growth, as both modes only differ in the initial step of substrate assimilation. An initial attempt to characterize growth under organoautotrophic conditions was performed and it was observed that the biomass yield was almost three times lower than using fructose as substrate for heterotrophic mode. Furthermore, growth under anaerobic conditions was explored as an alternative to avoid the potential explosion risk from mixtures of hydrogen and oxygen. It was found that respiration, either using oxygen or nitrate as electron acceptor, is needed for ATP production in *C. necator* and thus to support growth. To facilitate growth and reoxidation of NADH also under anaerobic conditions a new strain was generated in which the ethanol fermentation pathway was introduced. A preliminary characterization of the fermentative capacity of this strain showed that no ethanol was produced when grown organoautotrophically.

# 1. Introduction

The microorganism *Cupriavidus necator* (also known as *Ralstonia eutropha*, and previously known as *Hydrogenomonas eutrophus* and *Alcaligenes eutrophus*) is a Gram-negative bacterium that belongs to the class β-*Proteobacteria*. Its natural habitat are soil and freshwater biotopes where transient anoxic conditions occur (Aragno 1992). Its genome is composed of two chromosomes and one megaplasmid (pHG1) that together codify for more than 6000 genes. The genome size is 7416 kb with 4052 and 2912 kb for the chromosome 1 and 2 and 452 kb for pHG1 (Pohlmann et al. 2006).

*C. necator* has been thoroughly studied as a model microorganism for production of polyhydroxyalkanoates, more specifically polyhydroxybutyrate (PHB). This storage molecule is produced when a carbon source is abundant, but other nutrients like oxygen, nitrogen or phosphate are limiting. PHB is a biobased and biodegradable polymer that could solve the environmental problems associated with petroleum-derived plastics that are extremely persistent in the environment. PHB is also favorable over conventional plastic as it can be produced from renewable resources such as sugar compounds.

*C. necator* has recently been described as a potential production platform for biofuels (Nybo et al. 2015). This is mainly due to its capacity to grow chemolithoautotrophically using $CO_2$ as the only carbon source and $H_2$ as an energy donor (Ishizaki & Tanaka 1990). The search for microorganisms capable of fixing $CO_2$ has been significant in the research community because of the concern for increasing $CO_2$ levels in the atmosphere caused by transportation and industry. Another reason for considering *C. necator* as a potential production platform for biofuels arises from its ability to store big quantities of PHB. Applying metabolic engineering strategies, the carbon flux going towards PHB production could be redirected towards products of interest. This has already been shown for production of isobutanol and 3-methyl-butanol (Li et al. 2012).

The establishment of hydrogen-oxidizing bacteria as biofuel producers would represent a valuable alternative compared to photosynthetic microorganisms. The need of being exposed to light represents a major drawback for photosynthetic microorganisms since they can only grow to limited biomass density in photobioreactors before light penetration in the culture is significantly reduced (self-shading effect). This is not the case for *C. necator* since no light is needed for its growth.

Under autotrophic conditions, $CO_2$ fixation in *C. necator* occurs via the Calvin-Benson-Bassham (CBB) cycle (Bowien & Kusian 2002). The ribulose-1,5-bisphosphate carboxylase/oxygenase (commonly known as rubisco) adds a molecule of $CO_2$ to a molecule of ribulose-1,5-biphosphate to obtain 3-phosphoglycerate. The latter is further metabolized through the central carbon metabolism (Figure 1). Rubisco, together with the rest of the enzymes needed for the pathway, are present in the genome as a *cbb* operon. Even though two copies of this operon are found, one on the megaplasmid (pHG1) and another one on chromosome 2, only the transcriptional activator *cbbR* in chromosome 2 is active. Hence, the expression of both *cbb* operons is controlled by the chromosomally located *cbbR* regulator (Pohlmann et al. 2006).

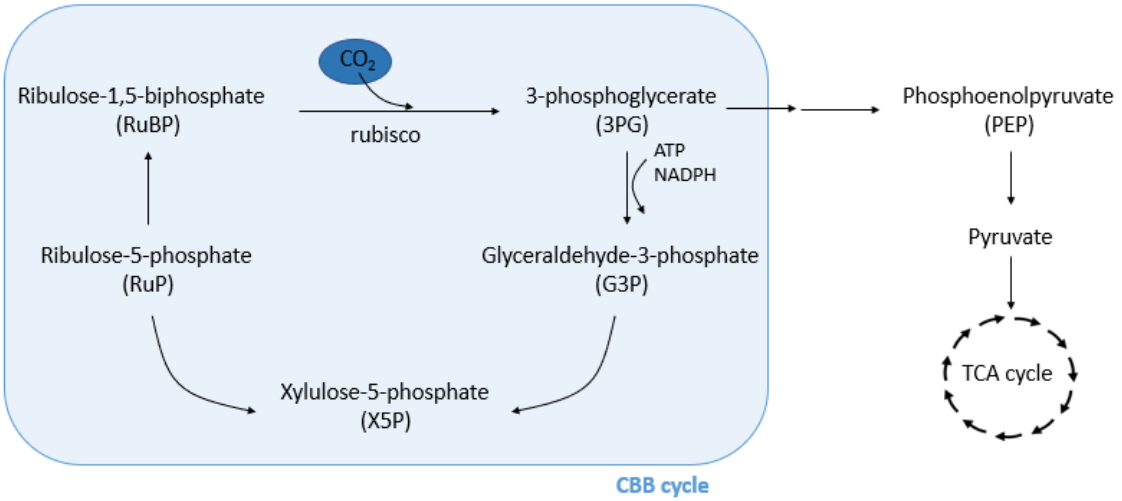


Figure 1. Calvin-Benson-Bassham cycle in *C. necator*.

The reducing power to drive the carbon assimilation/reduction is obtained from hydrogen, which is assimilated by hydrogenases that formally oxidize molecular $H_2$ into 2 $H^+$ and 2 $e^-$ and transfer the electrons to form NADH/NADPH. There are two types of hydrogenases in *C. necator*: membrane bound hydrogenases (MH) and soluble hydrogenases (SH). The MH is associated with the respiratory chain so it ultimately leads to ATP production. SH are found in the cytoplasm and can bind cofactors like $NAD^+$ or $NADP^+$ to obtain reduced cofactors. These SHs are bidirectional depending on biological conditions, such as partial pressure of $H_2$or NAD/NADH ratio (Nybo et al. 2015). The schematic cellular structure and reactions can be seen in Figure 2.

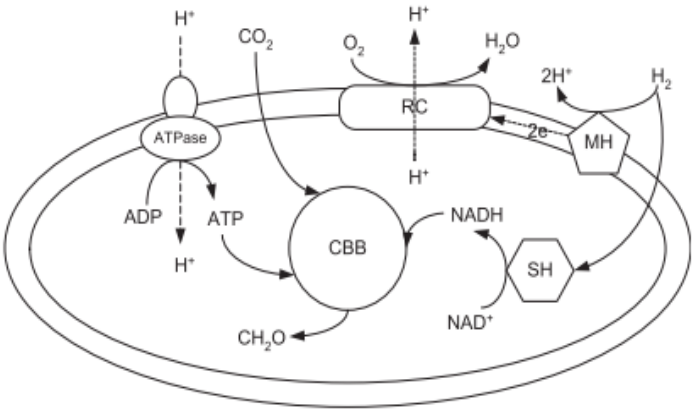


Figure 2. Schematic cellular structure and reactions involved in autotrophic growth (Yu et al. 2013).MH, membrane bound hydrogenase; SH, soluble hydrogenase; RC, respiratory chain; CBB, Calvin-Benson-Bassham cycle.

The CBB cycle is a highly energy demanding pathway since in order to assimilate one $CO_2$, 2.67 ATP and 1.67 NADH are needed (Grunwald et al. 2015). This implies that a high flux of $H_2$ is needed during chemolitoautotrophic cultivation to meet the energy demand. However, one should keep in mind that a gas mixture containing $H_2$ and $O_2$ could lead to an explosion if mixed in the wrong concentrations. The lower and upper explosion limits in $H_2$/air mixtures at atmospheric pressure are 3.6-4.2 mol% and 75.1-77 mol% depending on the performed test (Schroeder & Holtappels 2005). This problem has been solved by working with pressurized explosion-proof reactors (Ishizaki & Tanaka 1990), however it requires specific equipment, which can be expensive. A safer way of generating the desired working conditions using regular equipment was successfully applied by Li et al (2012) where the $H_2$ was produced externally by

an electricity-powered hydrogen generator on demand and fed into the bioreactor, thus reducing the risk of explosion by avoiding the storage of $H_2$ gas.

A different approach to reduce the risk of explosion is to grow *C. necator* under anaerobic conditions. It has been shown that high concentrations of $O_2$ (35 mol%) cause growth inhibition whereas lower concentrations (23 mol%) are more suitable (Yu et al. 2013). Furthermore, it is known that *C. necator* is capable of growing under anaerobic conditions using nitrate or nitrite as electron acceptor instead of oxygen (Pfitzner & Schlegel 1973). Parts of the denitrification pathway including the nitrate reductase and nitric oxide reductase are encoded both on chromosome 2 and the megaplasmid (pHG1) whereas other genes necessary for the pathway are unique for either chromosome 2 or the megaplasmid (pHG1) (Pohlmann et al. 2006). During the denitrification process two different growth phases can be observed: first, nitrate is converted into nitrite which is accumulated and then nitrite is consumed and transformed into atmospheric $N_2$. However, this process is not efficient since the maximum growth rate observed when grown under anaerobic chemolithoautotrophic conditions was 0.509 $d^{-1}$ (or 0.021 $h^{-1}$) (Tiemeyer et al. 2007). This drastic effect was unexpected since usually, maximum specific growth rates are reduced by only 20 % when using nitrate instead of oxygen as electron acceptor (Hulsbeek et al. 2002). The decrease in growth rate is most probably due to the accumulation of intermediate toxic compounds such as nitrite ($NO_2^-$) generated during denitrification.

Another alternative to canonical autotrophic growth on $CO_2$ and $H_2$ is to use formic acid as both the energy donor and carbon source. This trophic mode is called organoautotrophic growth and has been recognized by researchers as a potential platform process for future bio-industry concepts (Yishai et al. 2016). The main advantage of formate as carbon and energy source is that it is safer and less volatile compared to $H_2$ and that formic acid can be produced using biological or physicochemical means. More precisely, formic acid can be obtained electrochemically from $CO_2$ and $H_2O$ (Udupa et al. 1971). It is then further oxidized by a formate dehydrogenase in the cell obtaining one NADH and one $CO_2$. At least four different formate dehydrogenases are found in *C. necator*'s genome (Pohlmann et al. 2006). The main formate dehydrogenase is a soluble heterotetrametric enzyme which is both molybdenum and NAD-dependent. It is encoded in an operon containing five genes *fdsG*, *-B*, *-A*, *-C*, *-D* on chromosome 1. Upstream to the operon, there is a transcriptional regulator *fdsR* (Figure 3) (Pohlmann et al. 2006).

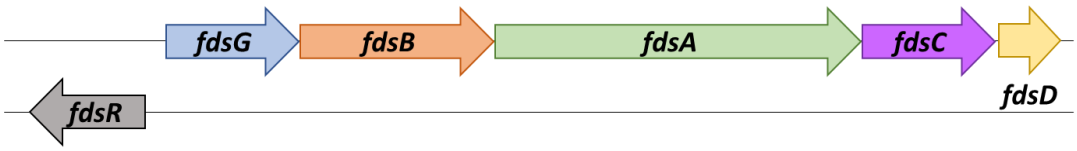


Figure 3. Fds gene cluster region in *Cupriavidus necator* H16. Adapted from Pohlmann et al. 2006.

In this way, the cell is considered to have a similar metabolic state since the $CO_2$ would still be transformed through the CBB cycle. The problem with this alternative to $H_2$ is that formic acid is toxic for the cells (Grunwald et al. 2015), so only low concentrations of it can be used.

Although much work was invested in the past to unravel the metabolic network/capabilities of *C. necator*, it has rarely been applied in a biotech context and only for the production of PHB. However, its unique capability of $CO_2$ fixation and $H_2$ consumption makes it an interesting organism for biotech processes driven by syngas (or other exhaust gas mixtures), but very little is known about the stability, biomass accumulation, or product yield under (organo-) autotrophic growth.

The aim of this project was therefore to establish stable continuous cultivations of *C. necator* under different growth conditions. Due to the inherent risk of working with hydrogen, chemolithoautotrophic growth was not studied. Instead heterotrophic and organoautotrophic growth modes were compared. Moreover, anaerobic conditions were applied to study possible applications of this alternative for future implementation of anaerobic chemolithoautotrophic cultivation. Following this line of work, a new strain was generated with the potential to ferment sugars under anaerobic conditions in order to obtain energy and regenerate cofactors.

# 2. Materials & methods

## 2.1. Strains, media and culture conditions

The strains and plasmids used and generated in this project are listed in *Table 1*.

Table 1. Plasmid and strains used in this project.

| Plasmid name | Relevant genotype | Reference |
|---|---|---|
| ΔNSI__Ptrc Adha Pdc | *Ptrc-AdhA-Pdc*; $Amp^R$ | Shabestary, unpublished |
| pLYK2-pBAD(TSS)-sgRNA(phac1) | *araBADp-sgRNA(phaC1)-Cas9-t;* $Km^R$ | Wang, unpublished |
| pLYK2-Ptrc(Adha-Pdc) | *Ptrc-AdhA-Pdc-t;* $Km^R$ | This project |
| | | |
| Strain | Relevant genotype | Reference |
| H16 | Wildtype | (Pohlmann et al. 2006) |
| RPF002 | pLYK2-Ptrc(Adha-Pdc) | This project |

The competent *E. coli* cells (strain NEB10-β, New England Biolabs) used for transformation and amplification of the plasmids were recovered from the freezer at -80 °C. Cells were grown in Lysogeny Broth (LB) medium containing 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl and 5 ml/l Tris-HCl (1M) pH 7.5. Cells were cultivated at 37 °C and 200 rpm. High efficiency transformation was performed following manufacturer's instructions (New England Biolabs, Protocol C3019). *E. coli* transformants were selected on LB agar plates with kanamycin (30 mg/l) grown overnight at 30 °C.

The minimal medium used for the cultivation of *Cupriavidus necator* consisted of 1.5 g/l $KH_2PO_4$, 4.475 g/l $Na_2HPO_4 \cdot 2H_2O$, 1 g/l $NH_4Cl$, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.0265 g/l $CaCl_2 \cdot 2H_2O$, 0.0012 g/l $FeNH_4$-citrate, 0.5 g/l $NaHCO_3$ and 2 ml of the Hoagland-Lösung solution (Schlegel et al. 1961). The Hoagland-Lösung solution standard composition is 0.288 g/l $H_3BO_3$, 0.03 g/l $CoCl_2 \cdot 6H_2O$, 0.08 g/l $CuSO_4 \cdot 5H_2O$, 0.008 g/l $MnCl_2 \cdot 4H_2O$, 0.176 g/l ZnSO4·$7H_2O$, 0.05 g/l $Na_2MoO_4 \cdot 2H_2O$ and 0.008 g/l $NiCl_2$ (Volova et al. 2013). $FeNH_4$-citrate and $NaHCO_3$ were separately added after autoclaving. The pH was adjusted to 6.8.

*C. necator* was cultivated at 30 °C and 200 rpm. The electroporation process described by Aneja et al., 2009, was used for transformation of *C. necator*. The transformants obtained were selected on LB agar plates supplemented with kanamycin (200 mg/l) at 30 °C to select for the pLYK2-Ptrc(Adha-Pdc) plasmid.

Restriction enzymes were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and chemicals and sugars were purchased from Sigma Aldrich (Missouri, USA).

PCR amplifications were performed using Phusion polymerase (Thermo Fisher Scientific, Waltham, MA, USA) if not otherwise stated.

## 2.2. Plasmid construction

The construction of the plasmid pLYK2-Ptrc(AdhA-Pdc) was obtained by the Gibson assembly method (Gibson et al. 2009). The *Ptrc-AdhA-Pdc* fragment consisted of two genes, the alcohol dehydrogenase from *Synechocystis PCC6803* (GenBank: BA000022.2) and the pyruvate decarboxylase from *Zymomonas mobilis* (GenBank: M15368.1), codon optimized for *Synechocystis PCC6803*, under the control of a Ptrc promoter. This fragment was amplified by PCR from the ΔNSI_Ptrc-AdhA-Pdc plasmid using DA172 and DA175 as primers (Table 2). The backbone vector was amplified from pLYK2-pBAD(TSS)-sgRNA(phaC1) using DA173 and DA174 as primers.

The PCR products were verified using agarose gel electrophoresis (0.8 % agarose concentration) and the correct bands were cut out and purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA). Both PCR products were treated with *DpnI* to degrade possible remaining plasmid template molecules and further purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA).

The two fragments, *Ptrc-AdhA-Pdc* and backbone vector, were ligated using the Gibson Assembly Master Mix (New England BioLabs, Ipswich, MA, USA) and incubated at 50 °C for 2 hours. The resulting product, pLYK2-Ptrc(AdhA-Pdc), was directly transformed into NEB 10-β competent *E. coli* cells from New England Biolabs.

Six isolated colonies were selected and the presence of the plasmid pLYK2-Ptrc(AdhA-Pdc) was verified by colony PCR using LUYA002 and LUYA386 as primers. Two positive clones were grown overnight at 30 °C in LB medium supplemented with kanamycin (30 mg/l).

Table 2. Primers used in this project.

| **Name** | **Sequence**[a] |
|---|---|
| DA172 | 5’ ttcatactcccgccattcagagATGAGCTGTTGACAATTAATCATCCG 3’ |
| DA173 | 5’ CTCTGAATGGCGGGAGTATGAA 3’ |
| DA174 | 5’ aactcctatagctaaacgccactagttcgactgcagTTATCAACTTGAAAAAGTGGCACC 3’ |
| DA175 | 5’ tggcgtttagCTATAGGAGTTTG 3’ |
| LUYA002 | 5’ ATTACCGCCTTTGAGTGAGCTGATA 3’ |
| LUYA386 | 5’ TGTTGCTCGAGGTGCCACCTGACGTCTAAG 3’ |
| KISH090 | 5’ CTACAGTCAACTTGAAGTTAGATTGGA 3’ |

[a] Lowercase sequences do not anneal to the template

The extracted plasmid was verified by restriction enzyme cleaving using *EcoR*I and *Pst*I and sequenced using the primers LUYA002, LUYA386 and KISH090. No mutations were found in the sequencing results.

## 2.3. *Cupriavidus necator* strain engineering

The wildtype strain, H16, was transformed with the plasmid pLYK2-Ptrc(AdhA-Pdc) according to the electroporation protocol described by Aneja et al. 2009. Transformants were selected on LB agar plates supplemented with kanamycin (200 mg/l) at 30°C.

Six isolated colonies were verified by diagnostic PCR using LUYA002 and LUYA386 as primers. Also, the extracted plasmid was verified by restriction enzyme cleaving using *EcoR*I and *Pst*I. Clone 3 was saved as RPF002 and used for further characterization.

## 2.4. Heterotrophic growth

### 2.4.1. Heterotrophic growth in turbidostat

A bioreactor of the type Multi-Cultivator MC-1000-OD (Photon Systems Instruments) was used to perform the cultivation in turbidostat mode. The Multi-Cultivator is equipped with eight tubular reactors with a working volume of 65 ml. The temperature was kept constant at 30 °C during the cultivation. In these bioreactors, mixing is achieved by aeration, in this case the overall aeration flow was set at 80 ml/min of air equally distributed to the tubes (=0.15 ml air/ml culture volume·min). The gas flow rate was controlled by a mass flow controller (LAMBDA Instruments, Baar, Switzerland). The $OD_{720}$ was measured online and the target $OD_{720}$ was set to 0.5. Six different fructose concentrations were analyzed: 5, 2, 1, 0.2, 0.05, and 0.01 g/l. The starting $OD_{720}$ of the cultivation was 0.15.

### 2.4.2. Heterotrophic growth in chemostat

The Multi-Cultivator MC-1000-OD (Photon Systems Instruments) was used to perform the cultivation in chemostat mode. Besides the previously mentioned characteristics, the pump in charge of the inflow of medium can be independently regulated for each channel allowing the application of different flow rates for each bioreactor. Four different dilution rates were applied using a fructose concentration of 0.05 g/l, i.e., 0.2, 0.15, 0.1 and 0.05 $h^{-1}$. The same four dilution rates were applied when the initial fructose concentration was increased to 1 g/l. Finally, when the fructose concentration of 0.5 g/l was used, a wider range of dilution rates was analyzed: 0.36, 0.33, 0.3, 0.24, 0.21, 0.18, 0.15, 0.11, 0.09 and 0.05 $h^{-1}$. Due to the limitation of eight tubes, the dilution rates 0.3, 0.33 and 0.36 $h^{-1}$ were applied on the same tube by increasing the flow rate in that channel gradually to find the critical dilution rate where wash-out of cells occurs.

The temperature was kept constant at 30 °C during the cultivation in all cases, and the overall aeration rate was fixed to 80-100 ml/min of air using a mass flow controller (LAMBDA Instruments, Baar, Switzerland) depending on the number of bioreactors in use.

The cells were allowed to initially grow in batch mode until they reached the stationary phase before applying the continuous flow of medium. The starting $OD_{720}$ was 0.15. Samples were taken once the steady state was reached (after five times of residence) for residual fructose measurements.

## 2.5. Organoautotrophic growth in chemostat

Cultivation was performed in chemostat mode as described before with the modification of using formic acid as substrate. The initial concentration used was 0.75 g/l. Initially, four different dilution rates were applied 0.2, 0.15, 0.1 and 0.05 $h^{-1}$ in duplicates; however, the bioreactors in which the initial dilution rate of 0.2 $h^{-1}$ was applied were gradually increased in flow rate to find the critical dilution rate.

## 2.6. Residual fructose measurement

The concentration of fructose present in the bioreactors during heterotrophic cultivation was measured according to the Fructose Assay Kit from Sigma Aldrich (Missouri, USA). Samples were

extracted from the bioreactor and placed on ice until they were centrifuged for 5 min at 6000 rcf at 4 °C. The supernatant was recovered and used directly for analysis or stored at -80 °C.

The samples were treated as described in the manufacturer's instructions and the absorbance at 340 nm was measured in a spectrophotometer (SpectraMax i3x, Molecular Devices, California, USA).

## 2.7. Ethanol quantification

The ethanol production of the wildtype strain and the engineered strain was measured by gas chromatography. For the initial determination of ethanol production, both the wildtype and the engineered strain RPF002 were grown on LB medium or minimal medium supplemented with 0.75 g/l of formic acid.

Samples were taken and centrifuged for 10 min at 14000 rcf at 4 °C. 100 µl of supernatant were recovered and used directly for analysis or stored at -80 °C covered in parafilm to avoid ethanol evaporation.

In the gas chromatography vial, 10 µl of 1000x diluted 1-butanol was introduced to be used as internal standard together with 80 µl of the sample and 80 µl of acetonitrile. The ratio between the area of ethanol's peak and the area of butanol's peak was used to calculate the concentration of ethanol in the sample.

## 2.8. Oxygen depletion

Oxygen depletion during chemostat cultivation was performed by reducing the concentration of oxygen through mixing the supplied air with $N_2$ gas. The cells were cultivated under heterotrophic conditions (0.5 g/l of fructose).

Oxygen depletion during batch cultivation was performed by cultivation in tightly closed shake flasks. The cultivations were done in organoautotrophic conditions (0.75 g/l formic acid) and samples were taken after 45 and 68 hours for growth, ethanol and PHB production measurements. The same procedure was performed adding 0.84 g/l of sodium nitrate to determine the effect of denitrification.

## 2.9. PHB visualization

A 100 µl sample of cell suspension was stained by direct addition of 5 µl of 1 % NileRed in DMSO and incubated for 1 minute at room temperature. Cells were then harvested by centrifugation for 2 minutes at 18000 rcf. The cell pellet was recovered and resuspended in 30 µl of water.

Phase contrast and fluorescence images were obtained using a Ti eclipse inverted research microscope with a 100x/1.45 NA objective (Nikon). Images were captured using NIS-Elements Advanced Research software and were processed with Fiji (ImageJ).

# 3. Results

# Part I - Optimization of cultivation conditions

## 3.1. Heterotrophic growth

A series of experiments were performed under heterotrophic conditions in order to study the maximum growth rate and substrate uptake rate/capabilities of *C. necator*. Initially, a cultivation was performed in turbidostat mode where different fructose concentrations were applied, ranging from 5 to 0.01 g/l. The goal of this experiment was to investigate the dependence of growth rate and substrate concentration that should theoretically follow Monod-like distribution. During such conditions, the substrate concentration becomes saturated and the growth rate does not longer increase, allowing you to derive $\mu_{max}$, and the substrate concentration at which the growth rate is half the maximum growth rate, Ks (Figure 4). This indicates the optimal substrate concentration and helps to avoid carbon limitation conditions.

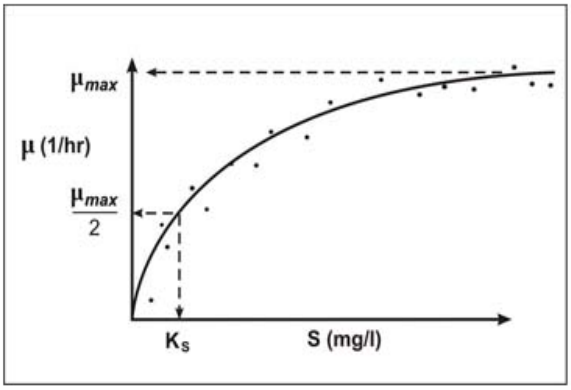


Figure 4. Example of Monod's relationship of maximum growth rate and substrate concentration. μ, growth rate; μmax, maximum growth rate; Ks, concentration at which the growth rate is half of the maximum growth rate; S, substrate concentration. Obtained from A. Cunningham, Center for Biofilm Engineering, Montana State University.

From the six concentrations tried, only those equal to or higher than 1 g/l were able to reach the target OD=0.5. In these bioreactors the growth rate at which the cells were growing was calculated and can be seen in Table 3. The three different fructose concentrations supported a similar growth rate around 0.2 $h^{-1}$.

Table 3. Calculated growth rate in turbidostat cultivation.

| **Fructose concentration (g/l)** | **Growth rate ($h^{-1}$)** |
|---|---|
| 5 | 0.20 ± 0.04 |
| 2 | 0.20 ± 0.05 |
| 1 | 0.23 ± 0.09 |

The small number of data points was not sufficient to obtain the Monod curve and turbidostat mode was not further pursued for future experiments, as it would not allow analysis of the differences in growth under varying substrate concentrations. Instead, chemostat mode was selected. At first, a fructose concentration of 0.05 g/l was chosen and several dilution rates were applied, ranging from 0.4 to 0.05 $h^{-1}$. The biomass over time can be seen in Figure 5 (panel A). The trend observed with the dilution rate 0.2 $h^{-1}$ suggests that the maximum growth rate

achievable for the cells under these conditions was lower than 0.2 $h^{-1}$. Furthermore, even though the cells were able to stabilize at lower dilution rates, after approximately 10 hours of cultivation they also got gradually diluted. This was believed to be a consequence of a carbon limitation situation where the fructose concentration was too low to support steady state growth.

The calculated mean $OD_{720}$ from the first 10 hours of cultivation shown in Figure 5 (panel A) were plotted versus the dilution rate. The higher the dilution rate the lower was the $OD_{720}$. However, according to the chemostat mass balance theory, the same OD should be reached under different dilution rates unless the applied dilution rate is higher than the maximum growth rate in which case wash-out will happen. This was indeed the case for this experiment as can be seen in Figure 5 (panel B).

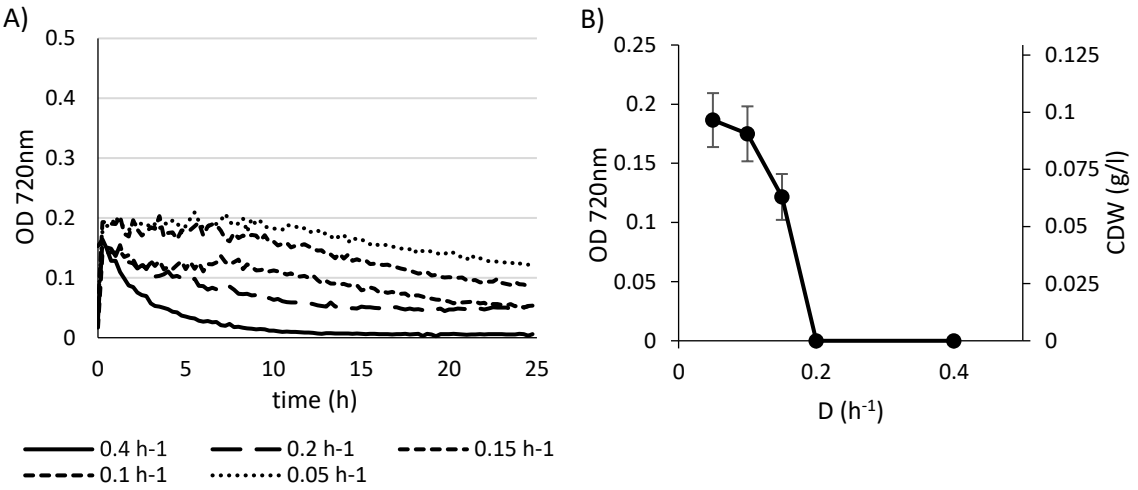


Figure 5. (A) $OD_{720}$ over time in chemostat cultivation with fructose concentration of 0.05 g/l. (B) Average biomass levels reached during the 10 first hours of cultivation at the different dilution rates.

In an attempt to avoid carbon limitation conditions, a new cultivation was started with an increased fructose concentration, 1 g/l. In this case, all bioreactors reached the same OD, around 0.8, except for the highest dilution rate, 0.26 $h^{-1}$, where the OD dropped to 0.6 (Figure 6, panel A). With the higher substrate concentration, a higher OD level could be reached as expected. Figure 6 (panel B) shows the expected chemostat plot where the OD reached is stable for low dilution rates and starts decreasing at 0.23 $h^{-1}$ where wash-out conditions begin to appear. Complete wash-out was not observed during this experiment as it can take very long when the difference between μmax and D is small. The residual fructose in the bioreactor was measured for the different dilution rates and, as expected, all the fructose was consumed at low dilution rates except at wash-out conditions. Then not all the fructose present in the medium was consumed by the cells.

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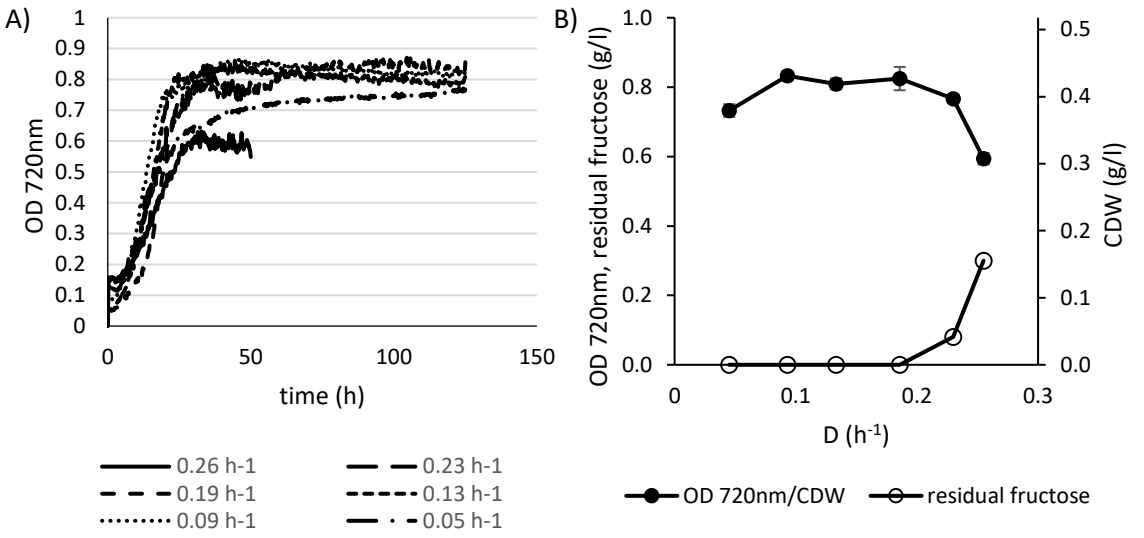



Figure 6. (A) $OD_{720}$ over time in chemostat cultivation with fructose concentration of 1 g/l. (B) Average biomass levels reached in steady state at the different dilution rates (●) and the residual fructose (○).

For technical reasons, this fructose concentration was not optimal for future experiments. The online OD measurements lose accuracy at ODs higher than 0.8 so it was decided to lower the fructose concentration to be in a more convenient OD range. Consequently, a new experiment with a fructose concentration of 0.5 g/l was started where also the culture medium was improved not showing precipitation (that was occasionally observed before) by fixing the pH to 6.8. A wider range of dilution rates was applied in order to find the rate where wash-out of cells occurs. An average value of $OD_{720}$=0.47 was reached at low dilution rates (Figure 7) and wash-out conditions were observed at dilution rates higher than 0.33 $h^{-1}$. The residual fructose for all dilution rates was measured, showing that fructose was completely consumed at low dilution rates whereas only 60% was consumed at a dilution rate of 0.33 $h^{-1}$.

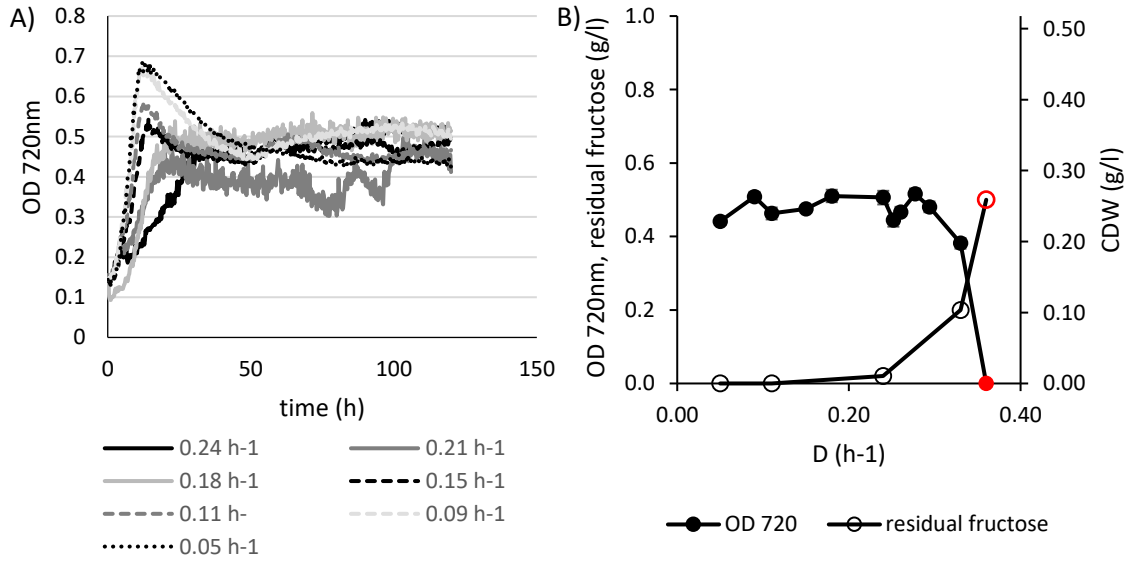



Figure 7. (A) $OD_{720}$ over time in chemostat cultivation with fructose concentration of 0.5 g/l. (B) Average biomass levels reached in steady state at the different dilution rates (●) and the residual fructose (○). Values marked in red are assumed values.

## 3.2. Organoautotrophic growth

The organoautotrophic growth was analyzed by running a chemostat experiment in the MultiCultivator. The concentration of formic acid needed to have the same mol carbon as when using 0.5 g/l of fructose was calculated.

$$\frac{0.5\ g\ fructose}{l} \times \frac{6\ C-mol\ fructose}{180\ g\ fructose} \times \frac{46\ g\ formic\ acid}{1\ C-mol\ formic\ acid} = 0.75\ g/l\ formic\ acid$$

This allows to compare both experiments. Also, high concentrations of formic acid have been shown to be toxic for the cells so low concentrations are needed.

Several dilution rates were tested for the determination of the maximum growth rate under these conditions. However, as it can be seen in Figure 8, this specific value was not found. The highest dilution rate applied was 0.32 $h^{-1}$ and the OD reached was 0.16, the same as the biomass observed for lower dilution rates. Since wash-out did not occur it can be concluded that the maximum growth rate exceeds 0.32 $h^{-1}$ for cells grown with 0.75 g/l of formic acid. For organoautotrophic growth, residual substrate concentration was not measured but as seen for fructose, all formic acid is expected to be consumed if the dilution rate is below μmax.

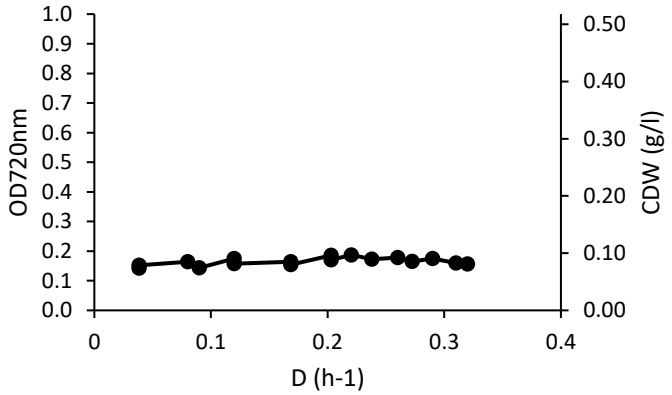



Figure 8. Biomass levels reached in chemostat cultivation with formic acid concentration of 0.75 g/l at different dilution rates.

## 3.3. Comparison of heterotrophic and organoautotrophic growth

If both trophic modes are compared, it can be seen that even though the concentration of substrate was equivalent in terms of C-mol/l, the biomass reached when using fructose was almost three times higher than when using formic acid.

Experiments using 0.5 g/l of fructose and 0.75 g/l of formic acid were compared by applying the Pirt equation (Figure 9). This equation is a linear model fitted towards the two variables substrate uptake rate and growth rate that were experimentally determined. The equation obtained for heterotrophic growth was:

$$q_S = 8.5051\ \times\ \mu + 0.0301$$

Whereas the equation for organoautotrophic growth was:

$$q_S = 1.9544 \times\ \mu + 0.0083$$

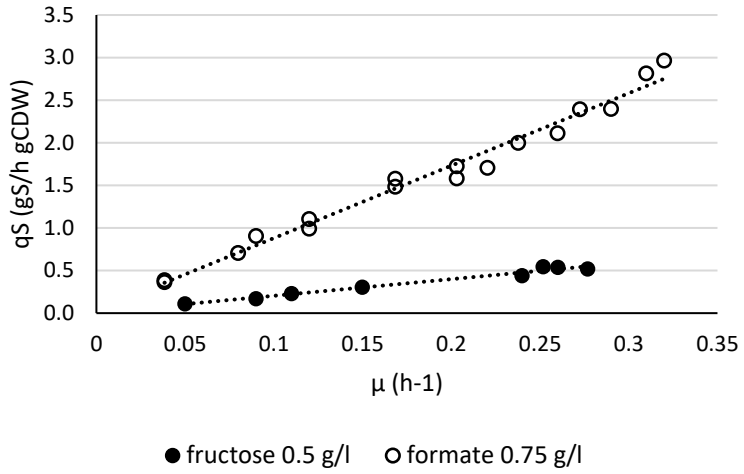



Figure 9. Pirt representation for heterotrophic growth (●) and organoautotrophic growth (○).

In the Pirt equation, the slope corresponds to the inverse of the biomass yield (g CDW/g substrate); the intercept, on the other hand, corresponds to the maintenance coefficient (g S/h·g CDW). In order to have a fair comparison, the obtained values were transformed into mol carbon (C-mol). The results can be seen in Table 4. As expected, the biomass yield was higher when the cells were grown under heterotrophic conditions. Also, the maintenance coefficient was higher for fructose than formic acid, which suggests that more energy is needed for non-growth cell processes when grown heterotrophically.

Table 4. Yield and maintenance coefficient obtained from the Pirt equation for heterotrophic and organoautotrophic growth.

| | **Yield (C-mol CDW/C-mol S)** | **Maintenance (g S/h·g CDW)** |
|---|---|---|
| **Fructose** | 0.63 | 0.030 |
| **Formic acid** | 0.23 | 0.008 |

# Part II – Oxygen depletion

## 3.4. Effect of oxygen depletion in maximum growth rate for the wildtype strain

A preliminary experiment to observe the effect of oxygen depletion in growth was performed. The wildtype strain was cultivated in chemostat in the MultiCultivator MC-1000-OD using 0.5 g/l of fructose in minimal medium. The concentration of oxygen supplied to the bioreactor was gradually decreased by mixing air with nitrogen gas. No nitrate was present in the medium. The growth rate was kept constant for every tube by fixing the applied dilution rate and the maximum growth rate supported by the new conditions was determined by the absence of wash-out in the tube. The obtained results can be seen in Figure 10. A dramatic decrease in growth rate was observed when the oxygen concentration was reduced suggesting a high demand of oxygen for high growth rates. Under completely anaerobic conditions, no growth was supported at the lowest fixed growth rate, 0.05 $h^{-1}$.

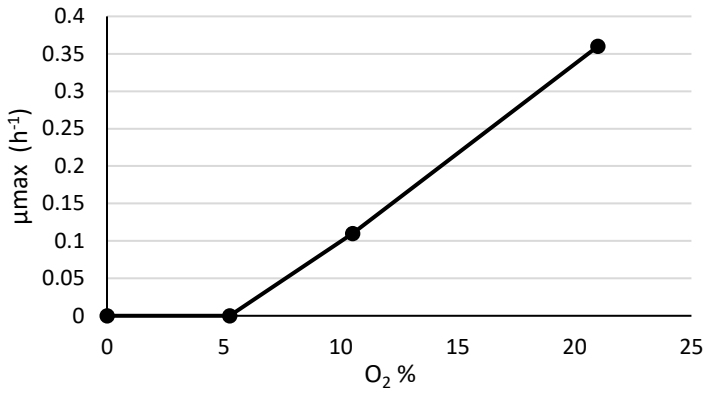



Figure 10. Maximum growth rate supported at different oxygen concentrations.

## 3.5. Heterologous expression of the ethanol fermentation pathway in *C. necator*

The ethanol fermentation pathway composed of an alcohol dehydrogenase and a pyruvate decarboxylase was introduced into *C. necator*. The activity of these enzymes was analyzed by measuring the ethanol production by gas chromatography. The cells were grown on Lysogeny Broth (LB) medium. The specific ethanol titer showed a 31.5 fold increase in the engineered strain compared to the wildtype (*Figure 11*). This suggests that the pathway was successfully introduced in the cell and that the enzymes were active and correctly expressed in *C. necator*.

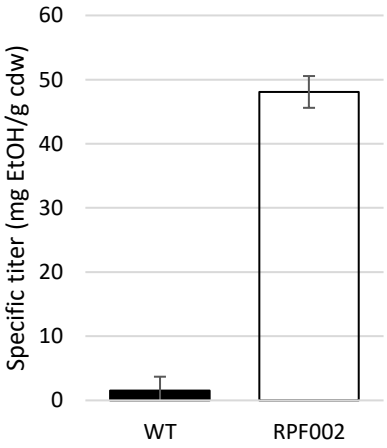



Figure 11. Calculated specific ethanol titer for the wildtype strain (black) and the engineered strain (white).

## 3.6. Shake flask microaerobic cultivation under organoautotrophic conditions

Cultivation of both the wildtype and the engineered strain RPF002 was performed in tightly closed shake flasks in microaerobic conditions. Initially oxygen was present in the flask, but it was assumed that it would be quickly consumed by the cells. Previous experiments showed that cultures in shake flask yield lower biomass than they do in a highly aerated bioreactor, presumably because of oxygen limited conditions.

Growth, ethanol and PHB production were measured to analyze the effect of the ethanol fermentation pathway. It was expected that a strain able to ferment could eventually regenerate $NAD(P)^+$ by excretion of EtOH as terminal $e^-$ acceptor.

Since *C. necator* can use nitrate as electron acceptor when oxygen is not present, the same experiment under microaerobic conditions was performed in parallel with the addition of

sodium nitrate. It was expected that denitrification could take place under these conditions and would help the cells to grow better. The results obtained can be seen in Figure 12.

The OD reached by the wildtype strain was almost double than the engineered strain when no nitrate was present to be used as electron acceptor in the denitrification process (Figure 12, panel A). However, in the presence of nitrate, the engineered strain RPF002 grew four times more than the wildtype strain reaching an $OD_{720}$ of 2.1 (Figure 12, panel B).

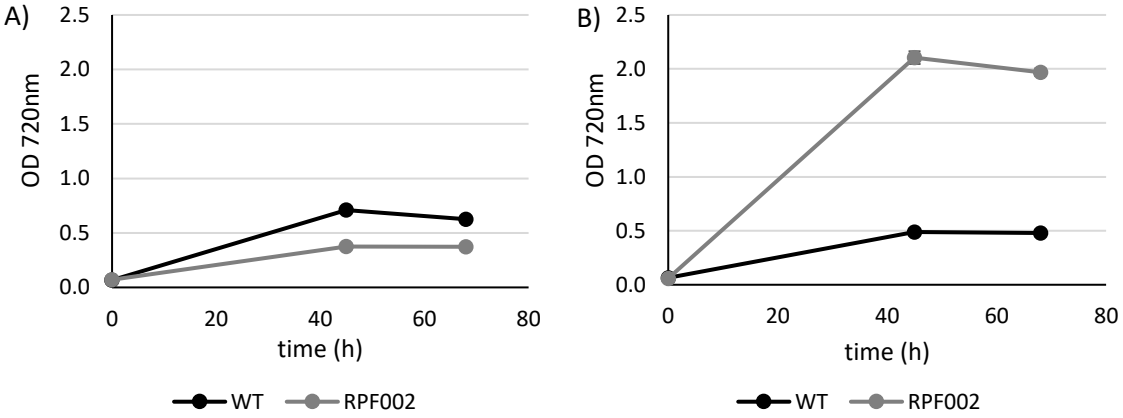
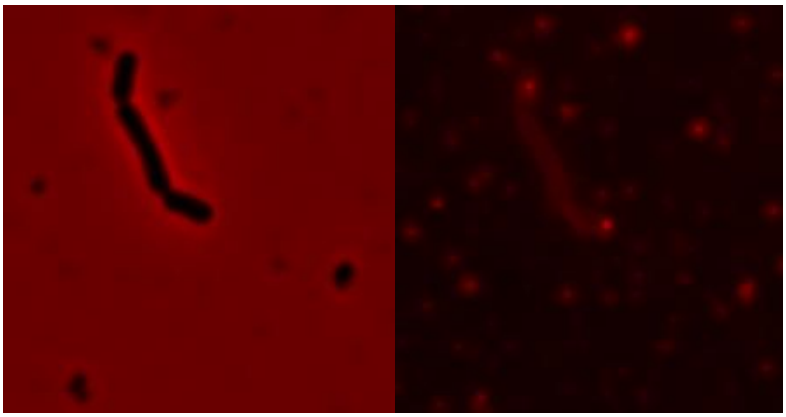



Figure 12. $OD_{720}$ over time during anaerobic cultivation in shake flask for both wildtype and engineered strain RPF002 without addition of nitrate (A) and with addition of nitrate (B).

No production of ethanol was detected throughout the cultivation in either strain, neither with nor without the presence of nitrate in the medium.

The samples taken for the cultures without addition of nitrate at 45 and 68 hours were visualized in a fluorescence microscope. However, it was hard to find cells where PHB granules could be detected for both strains. Two examples of isolated cells are shown in Figure 13. Two PHB granules can be observed for Figure 13, panel A whereas several can be seen in Figure 13, panel B. These were specifically chosen cells where PHB granules could be observed, yet the vast majority of the cells showed no granules concluding that PHB production was very low under these conditions. No significant differences were observed between the strains.

A)



B)

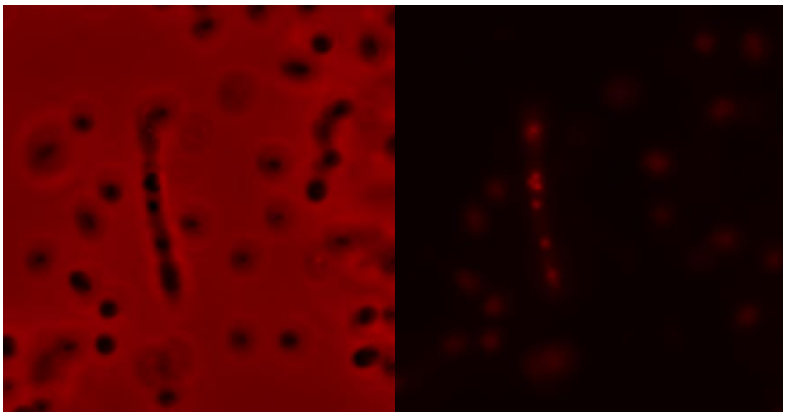


Figure 13. Phase contrast and fluorescence captions for visualization of PHB granules in cultures grown under organoautotrophic and microaerobic conditions. The cells were stained with NileRed that should bind to lipids such as PHB. Both panel A and panel B correspond to the wildtype strain.

# 4. Discussion

The metabolically versatile soil bacterium *C. necator* can assimilate a range of organic carbon sources, but interestingly, glucose is not among them. Instead, fructose is the substrate of preference for *C. necator* when grown under heterotrophic conditions. Systematic studies of growth under different carbon sources or growth modes are rare for *C. necator*. In this thesis, an attempt was made to analyze growth rate, biomass formation and substrate uptake in order to find the optimal substrate concentration for these specific conditions. For heterotrophic growth using fructose, it was found that 0.5 g/l of fructose was sufficient to support growth in steady state and sustain an OD within the linear range of the OD sensor incorporated in the bioreactor ($OD_{720}$=0.5).

During the experiments, a higher biomass was reached when higher substrate concentrations were provided as expected. However, the same correlation was not found for the maximum growth rate. In this project, the maximum growth rate observed for cultivation using 1 g/l of fructose, 0.23 $h^{-1}$, was actually lower than when 0.5 g/l of fructose were used. This could be explained by the medium precipitation problem encountered since the cells grown with 1 g/l of fructose could be facing nutrient limitation, thus leading to an observed apparent maximum growth rate lower than the real maximum growth rate.

Organoautotrophic growth was established by providing 0.75 g/l of formic acid, which was enough to support steady state growth and obtain an OD within the linear range of the OD sensor. Even though this concentration was equivalent to 0.5 g/l of fructose in C-mol/l, the OD reached in this case was almost three times lower. This difference was further studied by using the Pirt equation (Figure 9). The biomass yields were calculated and the difference between heterotrophic and organoautotrophic growth was confirmed. The biomass yield for fructose was again almost three times higher than for formic acid. This result was expected since the degree of reduction for both substrates per carbon is considerably different, 4 for fructose and only 2 for formic acid. When cells are grown under organoautotrophic conditions they cannot get the same reducing power per C-mol of substrate, which limits its growth capabilities.

$$Degree\ of\ reduction\ for\ fructose\ per\ carbon:$$

$$CH_2O \rightarrow 1 \times 4 + 2 \times 1 + 1 \times (-2) = 4$$

$$Degree\ of\ reduction\ for\ formic\ acid\ per\ carbon:$$

$$CH_2O_2 \rightarrow 1 \times 4 + 2 \times 1 + 2 \times (-2) = 2$$

Once stable cultivations were achieved for both trophic modes, a different approach was studied. Even though *C. necator* can be grown using $CO_2$ and $H_2$ as the sole carbon and energy source, respectively, the application of such conditions is complex due to the explosion risk of gas mixtures from oxygen and hydrogen. Thus, cultivation under anaerobic conditions would allow a reduced investment in bioreactor instrumentation and safety measures. As a first attempt, *C. necator* was grown under heterotrophic conditions and when the oxygen supply was gradually reduced to zero, no growth was observed (Figure 10). This could be because the lowest growth rate studied was 0.05 $h^{-1}$ and possible lower growth rates would be missed. However, *C. necator* has been shown to be a strictly respiratory microorganism and since no oxygen nor nitrate were provided, respiration was not possible. This would confirm the previous conclusion where no growth was obtained without respiration. In absence of an electron acceptor, no ATP can be obtained through the respiratory chain.

Alternatively, other microorganisms like *E. coli* are capable of obtaining ATP through substrate level phosphorylation. An essential enzyme for this pathway was identified as pyruvate-formate lyase (Pfl) as mutants lacking this enzyme were not able to grow under anaerobic conditions (Hasona et al. 2004). This enzyme catalyzes the degradation of pyruvate into acetyl-CoA and formate. In the case of *C. necator* this enzyme has not been detected (Pohlmann et al. 2006). The genome of *C. necator* was searched on NCBI and no annotation for this gene was found. It was also compared with the genome of *E. coli* using BLAST and no hit was found for the pyruvate-formate lyase. The absence of this enzyme could explain why no growth is supported when respiration is not available.

Another consequence of growing under anaerobic conditions is that NADH would accumulate without the possibility of being reoxidized to $NAD^+$ in the respiratory chain. To mitigate this problem, an engineered strain was generated where the ethanol fermentation pathway was introduced. Oxidized $NAD^+$ is then regenerated by conversion of acetaldehyde into ethanol.

The activity of this pathway was initially confirmed in the engineered strain with a 31.5 fold increase in specific ethanol titer (Figure 11) under heterotrophic conditions (growth on LB medium). However, no ethanol production was found in experiments that were carried out under organoautotrophic conditions. The biomass levels obtained under organoautotrophic conditions were relatively low, which made detection of a significant ethanol titer difficult. It is possible that the ethanol concentration was too low to be detected in the gas chromatography rather than that the cells were not producing ethanol at all. Furthermore, the cultures that showed production of ethanol were grown on LB medium whereas the following experiments used minimal medium. It could be possible that some components present in LB medium such as amino acids would increase the pool of pyruvate, a precursor for ethanol production. Another possibility is that the cells are actually consuming the previously produced ethanol under organoautotrophic growth. It has been shown that the alcohol dehydrogenase activity needed to metabolize ethanol is increased in *C. necator* when ethanol is added to the culture (Obruca et al. 2010).

In terms of growth, the engineered strain RPF002 showed lower final biomass concentration than the wildtype strain when no nitrate was present under microaerobic conditions (Figure 12, panel A). This could be because cell resources are being used for expression of the introduced plasmid and thus are not available for cell growth, or that the heterologous proteins somehow interfere with the metabolism of the cells. However, since ethanol production was not detected more experiments are needed to clarify. Interestingly, the engineered strain RPF002 grew better than the wildtype strain when nitrate was supplied. The final $OD_{720}$ was higher than any other value obtained during batch cultivation so an estimation of the maximum theoretical $OD_{720}$ achievable under these conditions was calculated. It was found that if all the C-mol from the substrate were transformed into biomass, a maximum $OD_{720}$ of approximately 0.8 was possible. Since the observed $OD_{720}$ exceed the theoretical maximum, it was concluded that cells may have consumed residual fructose or internal PHB accumulated from the precultivation. The wildtype strain on the other hand, grew worse when nitrate was present in the medium. This is not surprising since during the denitrification process toxic compounds such as nitrite are produced and could be killing the cells.

Regarding PHB production for the fermentation knock-in mutant and the wild type, some granules were indeed observed which confirms that the PHB staining procedure was successfully performed. Nevertheless, the number of granules per cell and the number of cells carrying granules at all was very low. *C. necator* needs to have excess of carbon source and growth-limiting conditions for some other compound such as nitrogen, phosphorus or oxygen. It was

considered that the cells were facing oxygen limiting conditions so that should not be the cause for lack of PHB production. More likely, the low carbon concentration and quality under organoautotrophic growth (0.75 g/l of formic acid) was the reason that the cells did not produce PHB.

This project was a first exploratory attempt into the different growth modes of *C. necator*. It was found that for heterotrophic chemostat cultivations, steady state growth was supported with a concentration of fructose of 0.5 g/l; However, although steady state growth was also achieved under organoautotrophic conditions (0.75 g/l of formic acid), the final biomass obtained in this case was lower. Furthermore, no growth was observed under fermentative conditions in wildtype *C. necator.* This is believed to be caused by the need of respiration for ATP generation. The results of this study opened avenues for future work including the introduction of the pyruvate-formate lyase into *C. necator* to enable fermentative growth. This would represent a step forward into the final goal of the project, performing autotrophic cultivations using $H_2$ and $CO_2$ under anaerobic conditions.

## Observations from this project that need follow-up

In general, the precultures were done on fructose even if they were going to be used for organoautotrophic cultivations with formic acid. It was decided to do it this way because of the toxicity of formic acid. Since precultures were done in batch, low concentrations of formic acid are needed to avoid toxicity levels, thus low biomass levels are achieved. Instead, when fructose is used higher biomass levels are achieved. However, this was sometimes problematic when transferring to new flasks. Residual fructose was suspected to be enough to support initial growth in the new flask giving false results. This was later avoided by washing the cells prior to transferring to new flasks to avoid transferring of the residual fructose. An alternative for the future would be to do fed-batch precultures directly with formic acid.

The engineered strain grew considerably slower than the wildtype strain even though no ethanol was detected in the GC measurements. One possible reason is that the cells are expressing the ethanol pathway but they consume it afterwards, so it is not detected in the GC. This is known to happen in yeast so it could be the case here as well, as it has been theorized by Obruca et al. 2010. Other experiments should be done to verify that the pathway is active, for example enzymatic assays.

Finally, even though the final $OD_{720}$ was not trustable it is possible that the ethanol pathway introduced increases the biomass yield on formic acid when nitrate is present. It could be that the ethanol production is some kind of “valve” for the cell to help cope with the toxicity of the compounds generated during denitrification.

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# Appendix

## Cell dry weight (CDW)

Several samples of different $OD_{720}$ levels were dried and weighed to obtain a correlation between $OD_{720}$ and cell dry weight. The result can be seen in Figure 14.

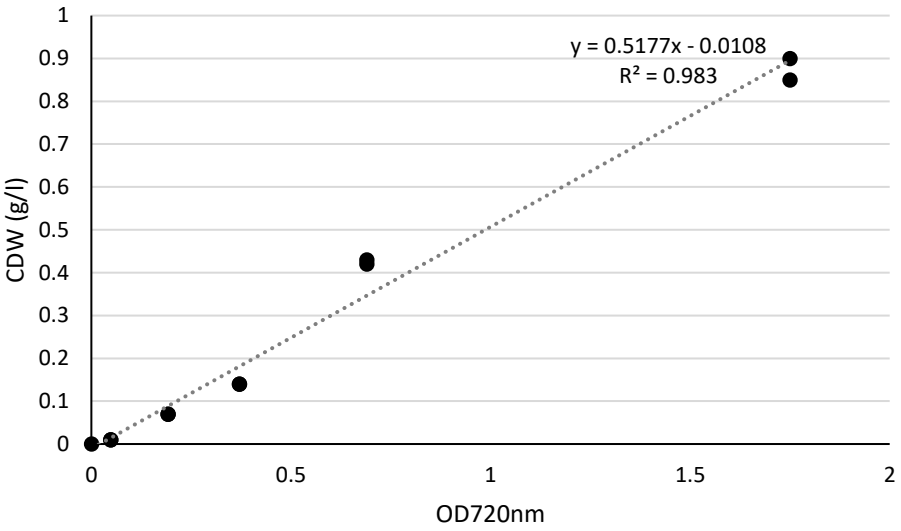


Figure 14. Correlation between $OD_{720}$ and cell dry weight (CDW).

## Pump calibration

After every chemostat cultivation, the real flow of each channel of the pump was measured. In Figure 15 an example of the correlation obtained can be seen.

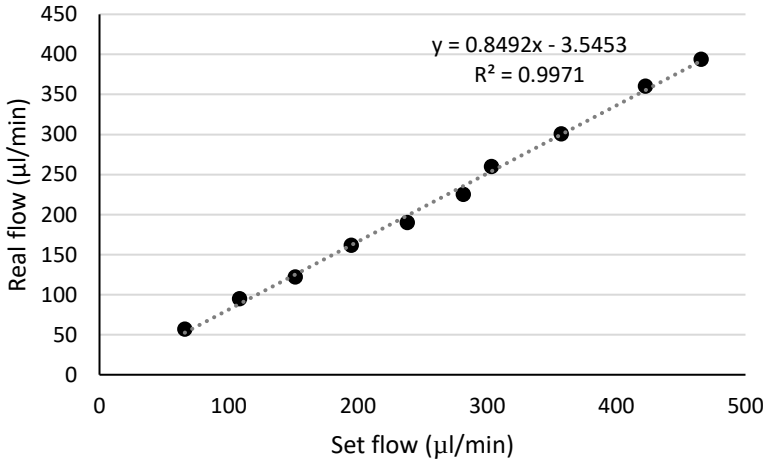


Figure 15. Correlation between the flow rate set in the pump and the real flow applied by the pump during a chemostat cultivation.