Adaptive laboratory evolution for enhanced performance of *Cupriavidus necator* on formic acid

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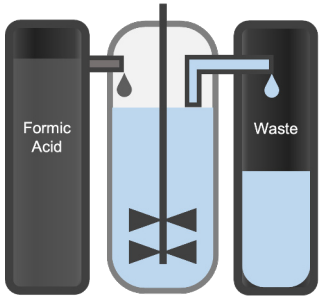
Introduction

CO_2 waste can be electrochemically reduced into formic acid, a soluble C1 molecule that can be used to store carbon and energy. *Cupriavidus necator (Ralstonia eutropha)* H16, a soil bacterium, capable of consuming and growing on formic acid as its sole source of carbon and energy, is well positioned to upgrade CO_2-derived formic acid into value added chemicals such as sustainable aviation fuels.

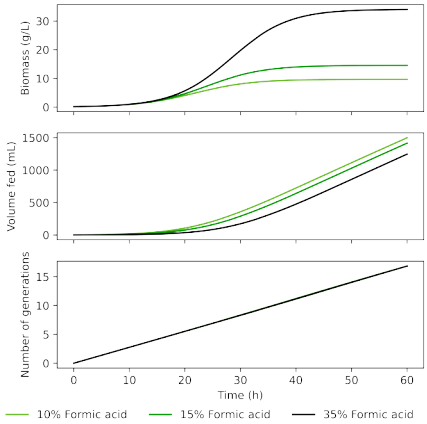
To improve the performance of C. necator on formic acid adaptive laboratory evolution (ALE), a proven tool for improving microbial fitness, has been conducted using continuous pH-stat bioreactors. The system works on the basis that consumption of formic acid raises the pH and triggers the addition of more formic acid to maintain the pH at 6.7, such that formic acid is provided at the same rate as it is consumed.



Continuous pH-stat bioreactor design

Modeling

The bioreactor design was modeled in Python together with governing equations for *C. necator* metabolism of formic acid using the genome scale model iCN1361. It was determined that the feed concentration of formic acid dictated the steady state biomass concentration. In pH-stat continuous fermentation, the media's feed rate is regulated based on pH. As a result, the system naturally adjusts the dilution rate to align with the microbial growth rate.

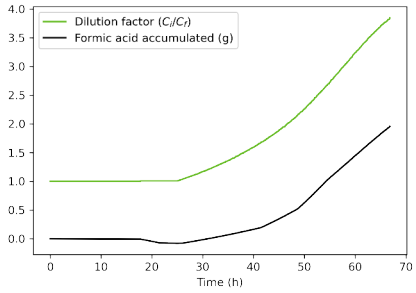


Model predictions for a pH-stat chemo-stat bioreactor for varying formic acid concentrations

Results: Initial Trials

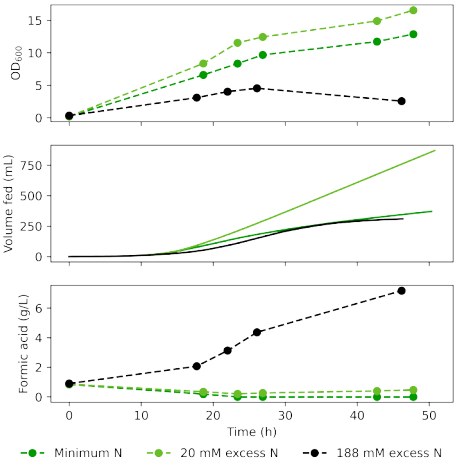
Sartorius 500 mL bioreactors were setup with pH control at 6.7 and level control set to 250 mL. The original bioreactor medium contains 20 mM sodium formate which was consumed triggering the addition of formic acid feed. The parental *C. necator* strain used for each fermentation was *C. necator* ATCC 17699 ΔH16_A0006 ΔpHG1 ΔphcA ΔphaCAB (CHC124).

It was discovered that formic acid accumulated during continuous fermentations, increasing to toxic levels for *C. necator.* Analysis of the data elucidated that formic acid accumulation was directly proportional to the dilution factor of the feed medium.



Comparing the accumulation of formic acid in the medium to the dilution factor of the medium

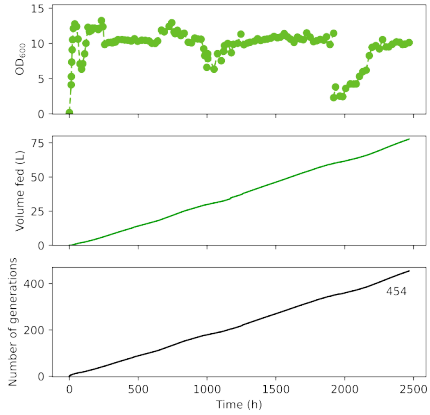
Residual ammonium hydroxide neutralized formic acid at pH 6.7 and this amount then accumulated. As the original batch medium was diluted by the feed medium the residual formate concentration changed from 20 mM to 188 mM, which is equivalent to the amount of residual ammonium in the medium. Using this to control the residual formate concentration feed medium was tested at the minimum nitrogen content and 20 mM excess nitrogen as demonstrated below.



Continuous pH-stat fermentations with varied amounts of NH_4OH in the 15% formic acid medium

Results: ALE

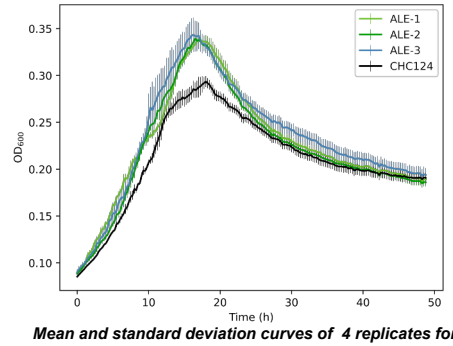
The ALE was run in three lineages started from a seed of CHC124. The feed medium contained 7.5% formic acid and the ammonium hydroxide concentration was altered to avoid formate accumulation. The ALE reactors have run for 2500 hours achieving over 400 generations in each lineage. The lineages were transferred into new bioreactors after 2000 hours for removal of biofilm formation.



An adaptive laboratory evolution lineage conducted in a continuous pH-stat bioreactor

After approximately 400 generations the lineages were drop plated onto LB and 15 µg/mL gentamicin agar to isolate colonies and select only for *C. necator* as it has natural resistance to gentamicin. Nine colonies were picked from each lineage. The isolates were run in a Bioscreen plate reader with the parental strain CHC124 on 60 mM sodium formate medium. The figure below shows the three most improved strains. These demonstrate a higher OD than the parental strain and the specific growth rates have improved by 12% with a 99% confidence interval.

These results are a good indication that beneficial mutations have occurred. To continue this work, whole genome sequencing will be done to identify potentially causative mutations. These mutations will be evaluated individually and in combination to identify those that improve growth on formic acid.



Mean and standard deviation curves of 4 replicates for each of the most improved strains

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