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

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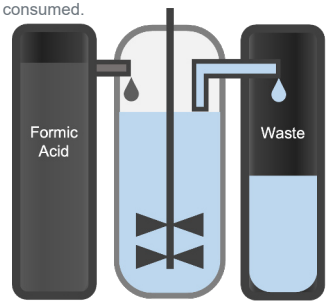
Introduction

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.

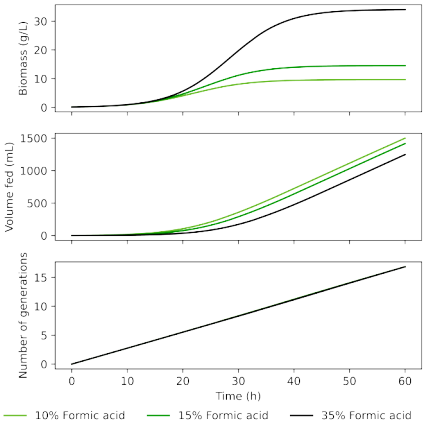
To improve the performance of *C. necator* on formic acid adaptive laboratory evolution (ALE), a proven tool for improving microbial fitness, has been conducted using continuous pH-stat bioreactors. The system works on the basis that consumption of formic acid raises the pH and triggers the addition 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.

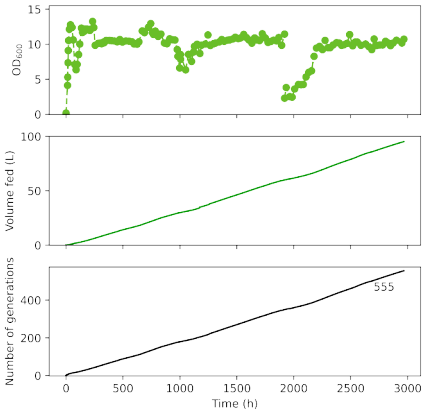


Model predictions for a pH-stat chemo-stat bioreactor for varying formic acid concentrations

Results

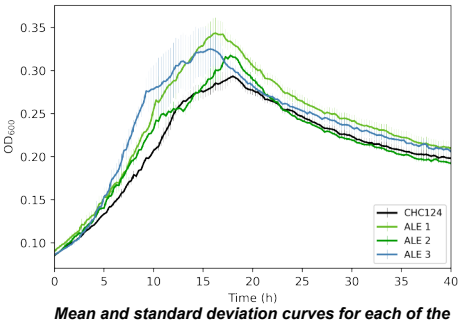
Sartorius 500 mL bioreactors were setup with pH control at 6.7 and level control set to 250 mL. The original bioreactor medium contains 20 mM sodium formate which was consumed triggering the addition of formic acid feed. The parental *C. necator* strain used for each fermentation was *C. necator* ATCC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

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 3000 hours achieving over 500 generations in each lineage. The lineages were transferred into new bioreactors after 2000 hours for removal of biofilm formation.



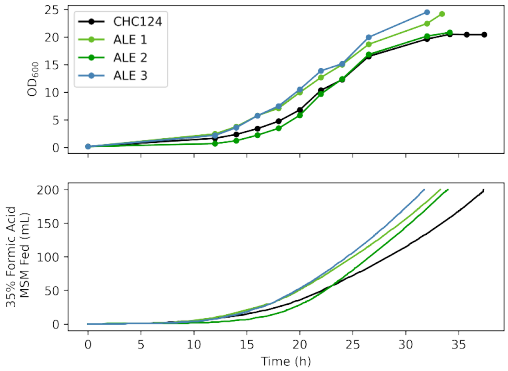
An adaptive laboratory evolution lineage conducted in a continuous pH-stat bioreactor

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 most improved strain from each lineage. These demonstrate a higher OD than the parental strain and the specific growth rates have improved as shown in the table with a 95% confidence interval.



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

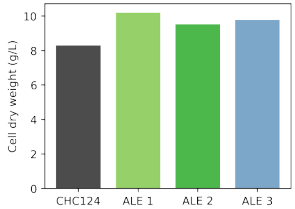
To characterize these strains in a process relevant environment the strains were grown on formic acid in pH-stat bioreactors. Each bioreactor started at a volume of 250 mL with an OD600 of 0.15. 200 mL of 35% formic acid MSM medium were feed to each bioreactor over the course of the experiment. The figure below shows the growth curves as well as the accumulative amount of formic acid fed over time. It can be seen from the OD600 growth curves, and the accumulative amount of formic acid fed that the ALE strains were able to consume the formic acid faster and grow to a higher OD600 than the parental strain CH124.



The growth and feeding curves on formic acid for the most improved ALE strains compared to the parental.

These improvements can be further seen in the table below of the calculated maximum specific growth rates and the figure below of the cell dry weights. These results indicated that the ALE strains have an improved growth rate and an improved yield of biomass.

Strain	Max growth rate (h^{-1}) (Plate reader)	Improvement (%) (Plate reader)	Max growth rate (h^{-1}) (Bioreactor)	Improvement (%) (Bioreactor)
CHC124	0.0901 (0.1088)	-	0.186	-
ALE 1	0.102	13%	0.221	19%
ALE 2	0.101	12%	0.261	40%
ALE 3	(0.132)	21%	0.220	18%



The cell dry weights from the end of formic acid pH-stat bioreactor runs

Conclusion

The ALE strains all exhibit improve growth rates and yield of biomass compared with the parental strain. Further research is being conducted to characterize the mutations the have caused these improvements.

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