Rational metabolic engineering of *Cupriavidus necator* to achieve sustainable biofuel and biopolymer production

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ABSTRACT

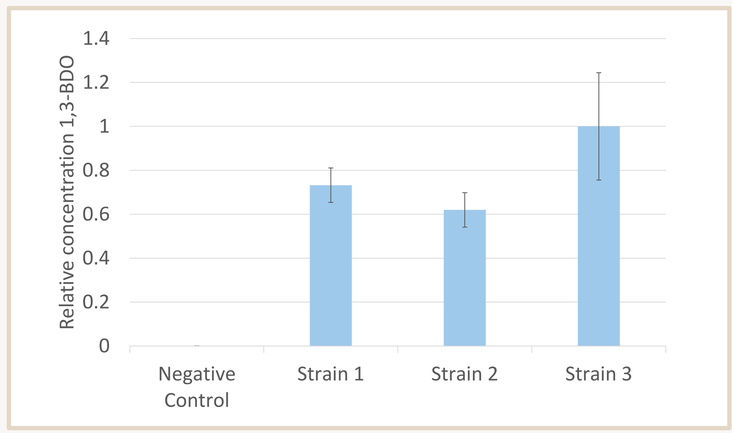
Today's chemicals industry faces growing concerns over emissions and energy consumption caused by the manufacturing of fossil-based products. To attain a decarbonised economy, microbial engineering offers promise by relying on renewable and sustainable resources instead. Novel biodegradable biopolymers can replace traditional polymers, while irreplaceable chemicals can be biologically produced, both options leading to reduced emissions. To this end, a microbial strain was developed to produce of Two platform chemicals: **1,3-Butanediol** (**1,3-BDO**) and **2-pyrone-4,6-dicarboxylic acid** (**PDC**). **1,3-BDO**, a valuable platform chemical and biofuel, is readily usable in the production of 1,3-butadiene, a precursor for various synthetic rubbers and plastics. The microbial chassis that was selected for fermentative production of 1,3-BDO is ***Cupriavidus necator***, an organism known for accumulating a high content of polyhydroxybutyrate (PHB). By rerouting its native PHB production pathway through the introduction of aldehyde reductase and butanal dehydrogenase encoding genes, successful 1,3-BDO production was achieved. Subsequently, various rational metabolic engineering strategies were devised to further enhance production, increasing metabolic flux towards the final product. Furthermore, it was also engineered to produce **PDC**, a promising building block for biodegradable biopolymers, paving the way to cost-efficient bio-based building blocks for novel polymers with a wide variety of applications.

A Rational engineering of a 1,3-BDO producing strain

Plasmid-based 1,3-Butanediol Production

To free up intermediates from the PHA pathway, *phaC1* was **knocked out.** Then, three different combinations of two enzyme variants were evaluated, encoding butanal dehydrogenase and an aldehyde reductase activities respectively. All test strains showed 1,3-BDO production.

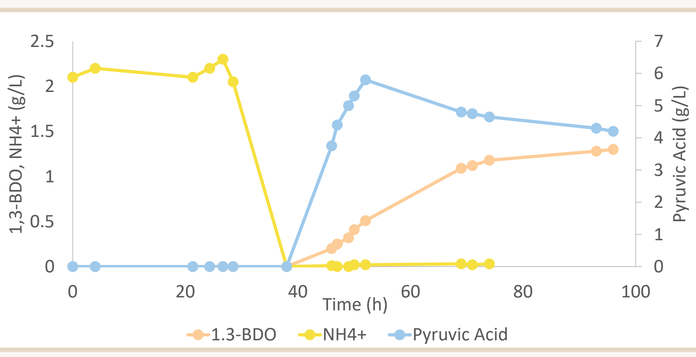
The results show a production difference of roughly **1.8-fold**, emphasizing need for **screening enzyme variants** for biosynthetic pathways.



Identifying Challenges

After subsequent genomic integration of the pathway in the *phaC1* locus, bioreactor experiments revealed a glaring **loss of carbon:** Secretion of **pyruvic acid**, a key intermediate in the 1,3-BDO biosynthetic pathway. This could have two reasons:

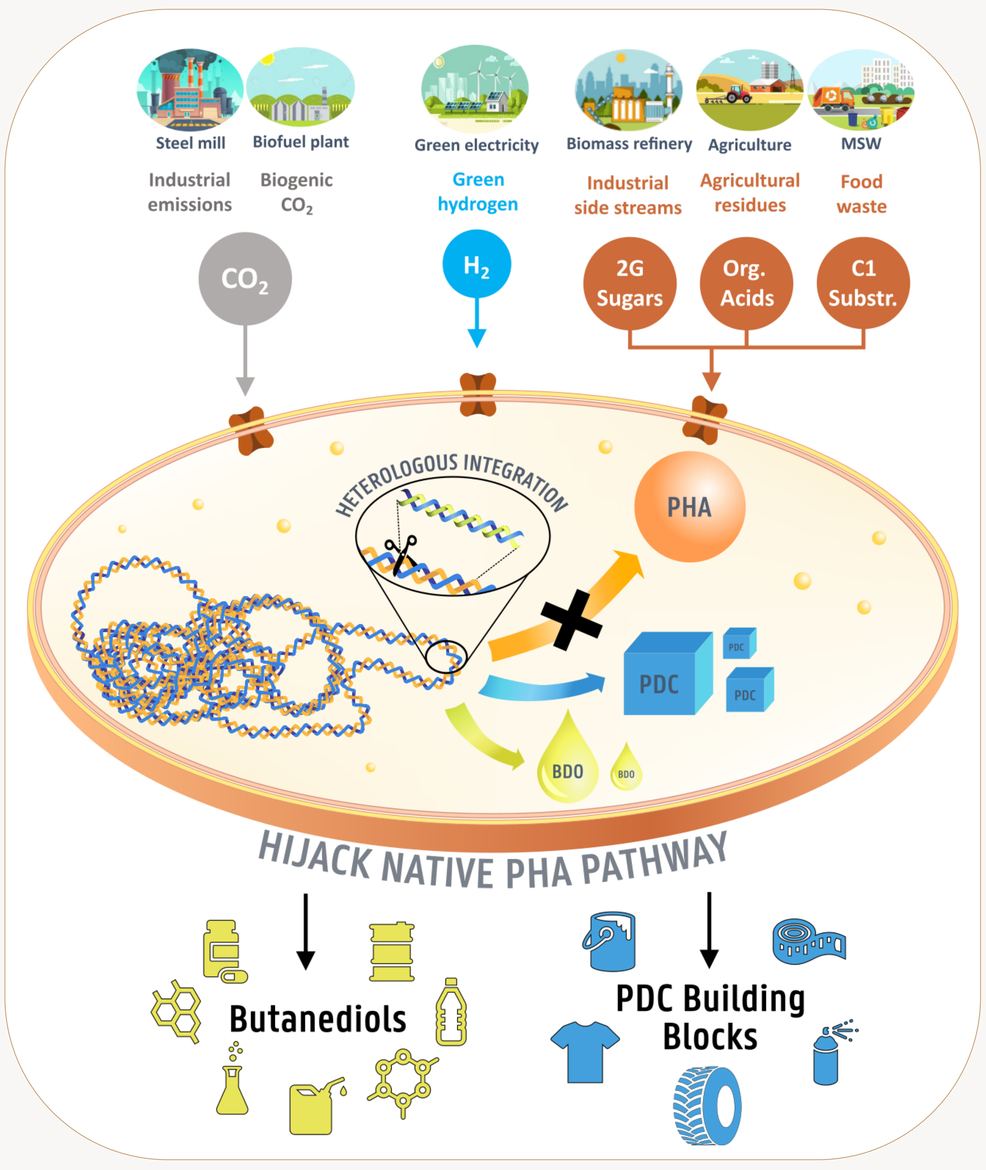
1. Insufficient enzymatic conversion
2. Lack of available co-factor NADH due to the new pathway



Strategy 1: Improving Transcription

Strategy 1 involves increasing transcription of the biosynthetic pathway by replacing the native *phaC1* promoter by a stronger one. Promoters (P_{j5}, P_{g25}, $P_{j5[C2]}$)that will be tested initially are chosen from literature[1].

- 1-fold expression — P_{phaC1} | 1,3-BDO pathway | *phaA* | *phaB1*
- 4-fold expression — P_{j5} | 1,3-BDO pathway | *phaA* | *phaB1*
- 6-fold expression — P_{g25} | 1,3-BDO pathway | *phaA* | *phaB1*
- 20-fold expression — $P_{j5[C2]}$ | 1,3-BDO pathway | *phaA* | *phaB1*

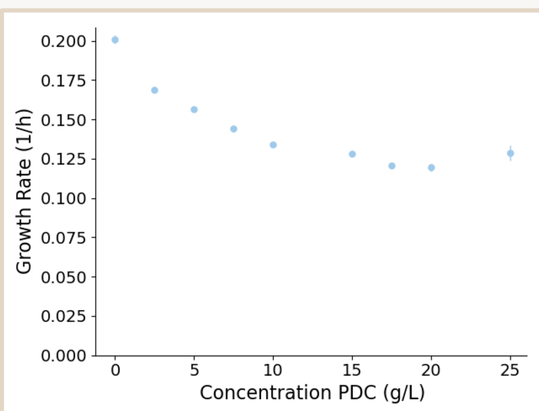


B Rational engineering of a PDC-producing strain

Toxicity

A first goal of the ECHO project was to test the toxicity of PDC on *C. necator.* The latter was grown on media with different PDC concentrations and optical density was measured:

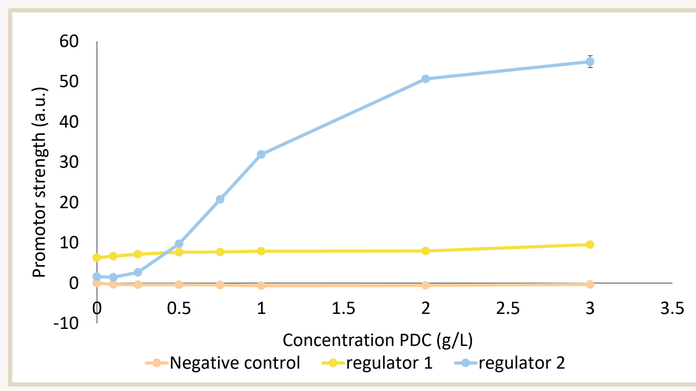
- Slight decrease on the growth rate at a low concentration (<5 g/L),
- **Similar growth rate** at higher PDC concentrations (10 to 25 g/L). This indicates that **PDC is not, or only mildly toxic for the host.**



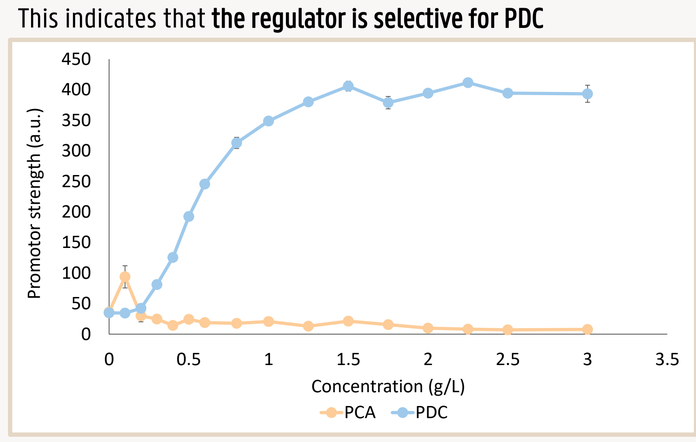
PDC-Responsive Regulators

PDC responsive regulators were tested for the purpose of coupling production of PDC to growth. Two different regulators were evaluated:

- regulator 1 shows no signal
- **regulator 2** shows a high intensity signal



The selectivity of the regulator towards the pathway intermediate (PCA) was tested → **no interaction shown** This indicates that **the regulator is selective for PDC**

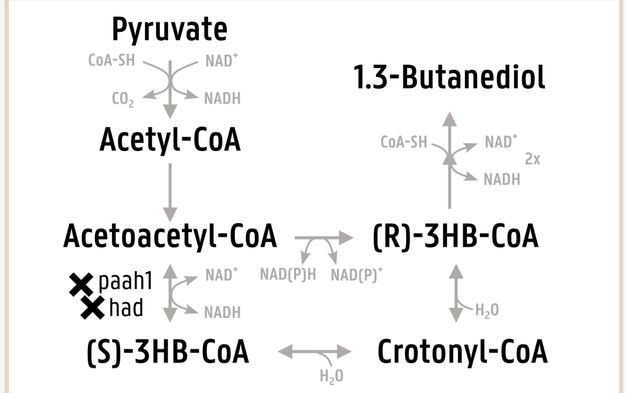


Strategy 2: Co-factor Utilization – Pathway Engineering

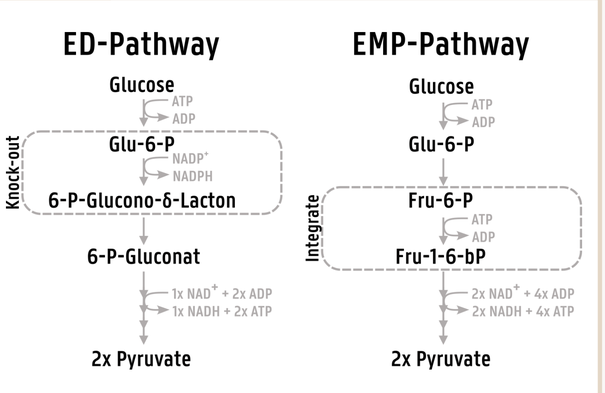
Two methods were devised to improve NADH accumulation, which are in development:

1. Deletion of Two NADH-dependent (S)-3-hydroxyacyl-CoA dehydrogenases – Tackles By-products and roughly 86% of NADH activity on acetoacetyl-CoA[2].
2. Replacement of the Entner-Doudoroff pathway by the Embden-Meyerhof-Parnas pathway – Production of 1 extra mole of NADH and ATP.

Method 1



Method 2



Proof-of-Concept PDC Production

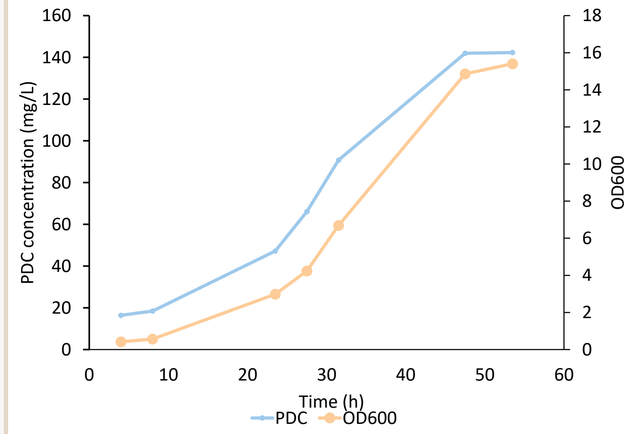
In order to establish proof of concept, a plasmid was built with the PDC production pathway. This plasmid was transformed into the *C. necator* and a production trial was performed with 60 g/L of glucose.

At 48 hours:

- **Optical density** (OD_{600}) reached a maximum value of **15.4**
- **PDC** reached a **concentration of 142 mg/L**

This indicates that the **production is coupled to growth**.

These results pave the way for further engineering of the microbial host as a high titer producer of PDC.



References

[1]Johnson, A. O., Gonzalez-Villanueva, M., Tee, K. L., & Wong, T. S. (2018). An engineered constitutive promoter set with broad activity range for Cupriavidus necator H16. *ACS Synthetic Biology*, *7*(8), 1918-1928

[2]Segawa, M., Wen, C., Orita, I., Nakamura, S., & Fukui, T. (2019). Two NADH-dependent (S)-3-hydroxyacyl-CoA dehydrogenases from polyhydroxyalkanoate-producing Ralstonia eutropha. *Journal of bioscience and bioengineering*, *127*(3), 294-300.

Acknowledgements

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