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Developing a CYP fusion protein screening platform for terminal hydroxylation of alkanes

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Introduction and Methods

Cytochrome (CYP) P450-Monooxygenases
CYPs are a diverse superfamily of heme-thiolate enzymes that catalyze the hydroxylation of chemically inactive carbon chains. This reaction involves transferring one oxygen atom from molecular oxygen to the substrate, while the other is reduced to water.[4] Enzymes can perform hydroxylation with high positional specificity. The **CYP153A** family is known for its ability to perform terminal hydroxylation especially on small molecules (e.g alkanes). It is hypothesized that the highly conserved motif, [NXXLLIVGGNDTT], found on the **I helix** within the protein core near the heme center, plays a critical role in facilitating this terminal hydroxylation.[1]

Electron transfer systems (ETs)
CYP153A proteins are dependent on electrons for their catalytic function. While these electrons originate from **NAD(P)H**, the CYP153A family cannot directly utilize NAD(P)H. Instead, they require dedicated ETs to mediate this transfer. A significant challenge in studying CYP153A enzymes is identifying their native ETs partners, as the genes encoding these systems are often not co-localized or part of the same operon. [3] In this study, electron transfer to CYP153A was facilitated by a ferredoxin reductase (**FdR**) and ferredoxin (**FD**) system from *Acinetobacter.* [2]

Linker design
Fusion proteins can be designed by fusing CYPs with their electron transfer (ET) systems using different linkers. This approach significantly simplifies the expression and purification of proteins and, moreover, often leads to a notable increase in catalytic activity due to more efficient electron transfer between the two protein domains. Recent studies have particularly emphasized the effectiveness of long, flexible glycine linkers.[5] [6]

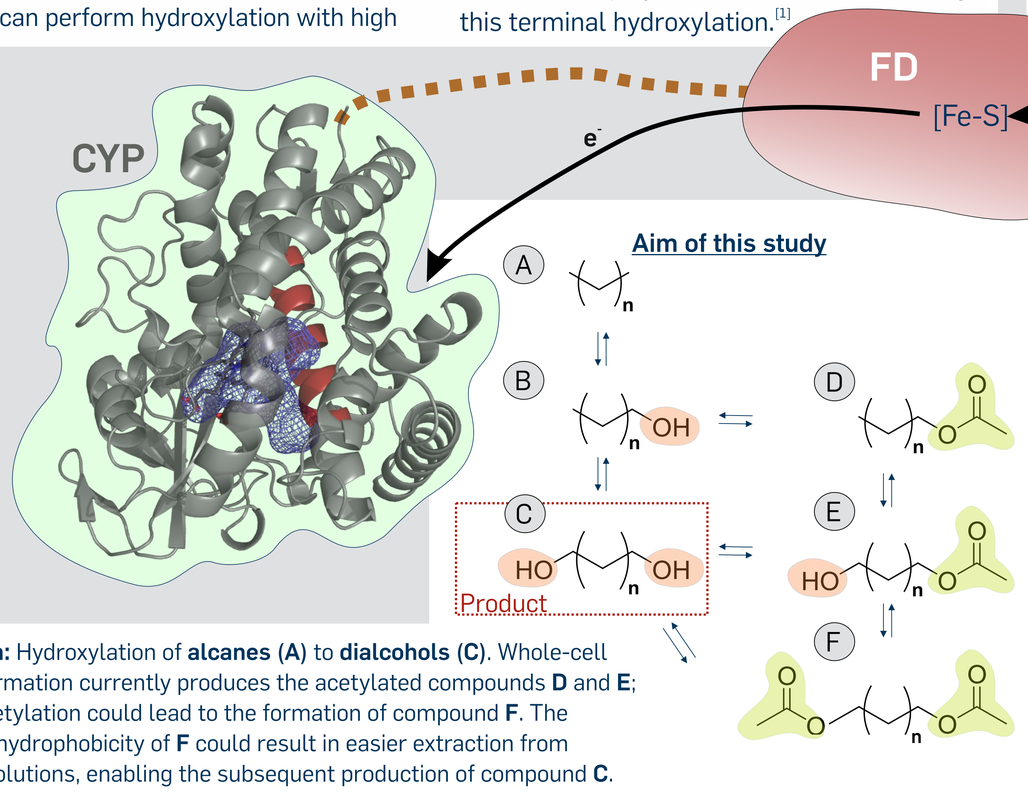

Fig. 1: Aim: Hydroxylation of **alcanes (A)** to **dialcohols (C)**. Whole-cell biotransformation currently produces the acetylated compounds **D** and **E**; further acetylation could lead to the formation of compound **F**. The increased hydrophobicity of **F** could result in easier extraction from aqueous solutions, enabling the subsequent production of compound **C**.

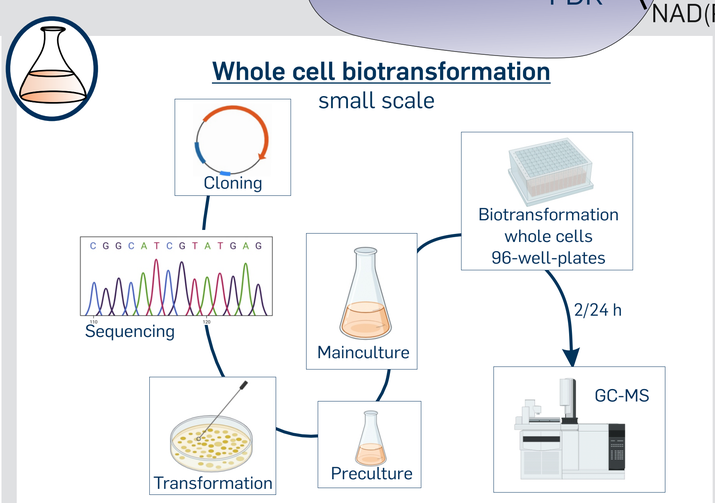

Fig. 2: Whole-cell biotransformation: The activity of different fusion proteins was tested; the most active protein is targeted for further modification.

Fusion-protein modification

Structure prediction of CYP

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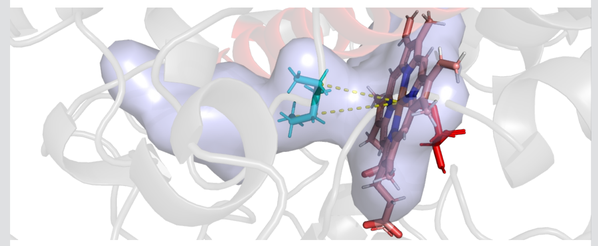
Identification of **substrate-binding pocket** and **interacting amino acids**
(Cavitomix and YASARA)

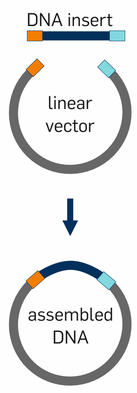


Results

Tab. 1: Overview of CYP153A Protein Constructs. Construction of eight different CYP fusion proteins and their controls using HiFi DNA Assembly (on the right). The left panel shows the specific linker combinations (4xGlycine and 2xHelix) utilized for each of the eight proteins. The control constructs consist of a fusion of the electron transfer (ET) domain without the CYP and only the CYP protein.

Nr.	Proteins	4Gly-4Gly	2Hel 2Hel	only CYP
1	CYP1	✓	✓	✓
2	CYP2	✓	✓	✓
3	CYP3	✓		✓
4	CYP4	✓		✓
5	CYP5	✓		✓
6	CYP6	✓		✓
7	CYP7	✓		✓
8	CYP8	✓		✓
9	CYP9	✓		✓

HiFi DNA Assembly



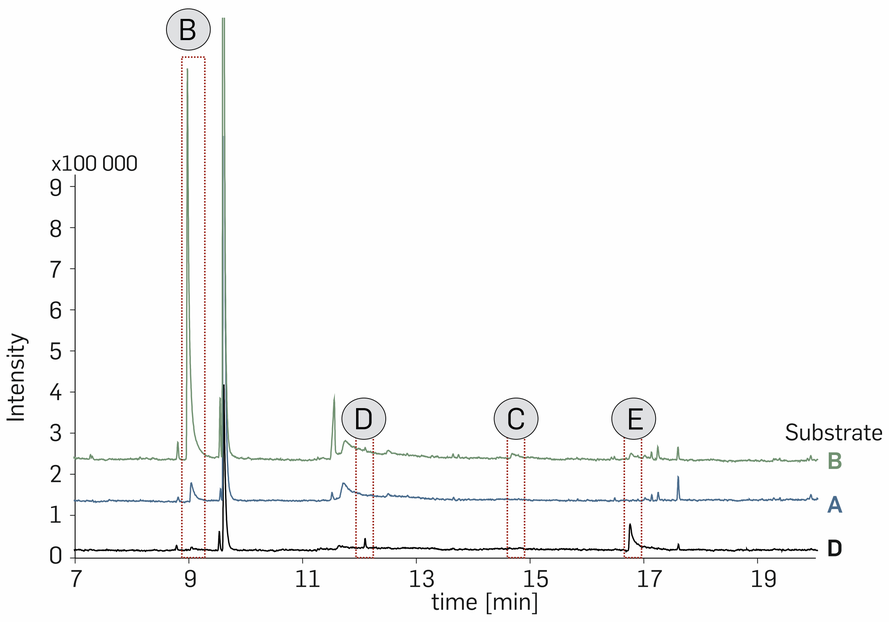


Fig. 3: GC–MS analysis of the 4Gly–4Gly–CYP2 construct after 24 h incubation with substrates B, A, and D. A slight conversion of B to D, C, and E was detected. Using A as a substrate led to the formation of B, whereas incubation with D resulted in the formation of E.

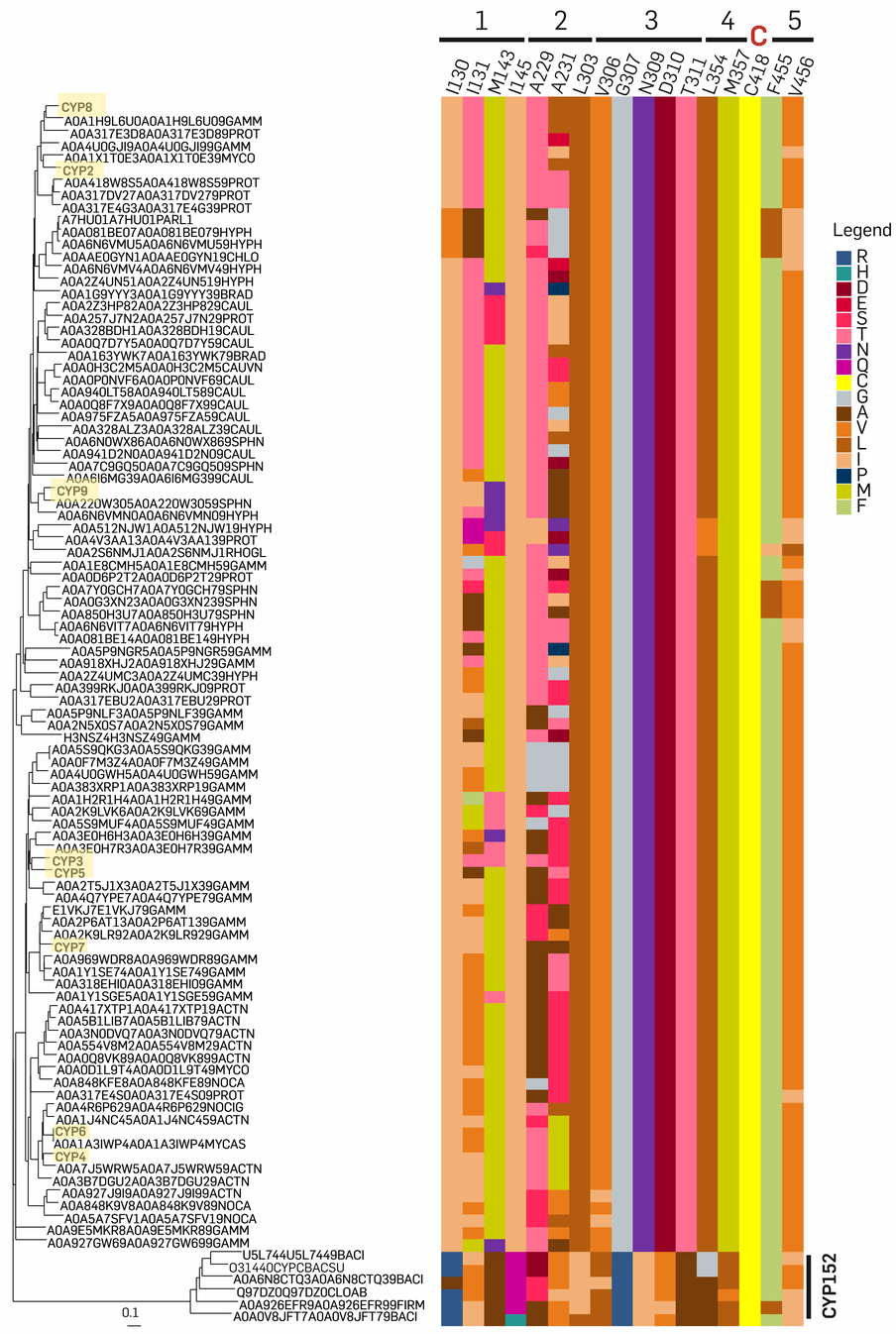


Fig. 4: The substrate binding residues within the active site of CYP153A proteins. The Fe-binding cysteine (**C**) is highly conserved in all CYP families. A unique conserved motif (partially in "4") is found exclusively in the CYP135A family. Interacting residues were identified using YASARA and Cavitomix. The conservation of residues varies; while some are highly conserved [4], others show some diversity within the CYP family tree shown. CYP152 served as an outgroup.

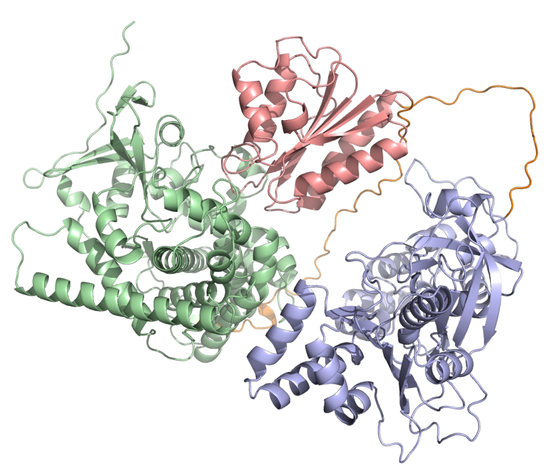


Fig. 5: Example of a natural fusion protein. Fusion proteins also occur naturally. This figure shows an example: a naturally occurring CYP fusion protein from the organism *Deinococcus aetherius* (A0A2I9DQ46).

Conclusion and Outlook

We have successfully established a small whole-cell biotransformation system that utilizes the organism's natural NADPH recycling function, enabling efficient and cost-effective product formation. This optimized reaction design enabled rapid screening of multiple protein fusion constructs. Our initial screening focused on FNR-FD-CYP153A fusion proteins linked by four glycine linkers, a linker design that had previously shown the highest activity. [6]

Looking ahead, we aim to scale up the reactions with the most active fusion constructs to achieve sufficient product yields for extraction. We also plan to pursue further protein modifications specifically targeting the **substrate binding** site and **linker design** to further improve the efficiency and specificity of the protein.

References
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