Improvement of PolyCYPs® Microbial Cytochrome P450 Cell Factory for Efficient Whole-Cell Biotransformation

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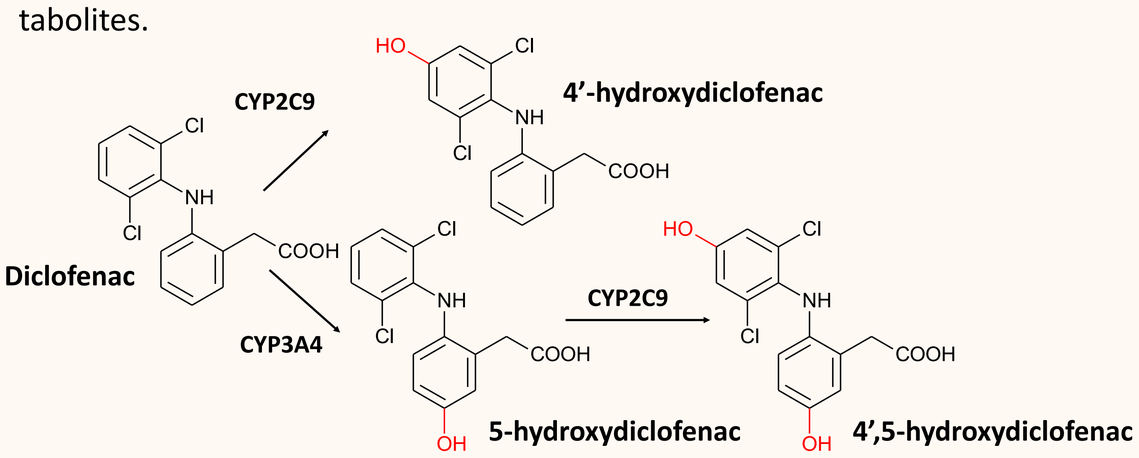
Abstract: PolyCYP6, a functional bacterial Class I cytochrome P450, was successfully mined from one of Hypha's actinomycete strains and converts the non-steroidal anti-inflammatory drug diclofenac into its hydroxylated human metabolites. Improvement of the PolyCYP cytochrome P450 cell factory for efficient whole-cell biotransformation was achieved through co-expression with specific redox partners and changing components of the T7 expression plasmid. The highest yielding recombinant strain expressed PolyCYP6 with the *P. putida* reductase system. An ampicillin resistant marker & high copy vector also further improved yields of hydroxylated products.

Background

Cytochrome P450 (P450) proteins are a superfamily of heme-containing thiolate enzymes and are known to participate in oxidative metabolism (such as hydroxylation, epoxidation, heteroatom oxidation & dealkylation) of approximately 70 to 80% of current drugs.[1]

Metabolites of drugs are often needed to be investigated and tested as part of regulated safety assessments during drug development. Non-selective and multi-step synthetic routes or incubation of compounds with low-yielding and high cost liver microsomes are frequently used for the production of drug metabolites. Whole-cell microbial biotransformation provides a cost-effective alternative with many advantages such as high selectivity, large scale application and cofactor regeneration.[2]

Diclofenac is a non-steroidal anti-inflammatory drug and is converted into the major 4'-hydroxydiclofenac and minor 5-hydroxydiclofenac metabolites by human CYP2C9 and CYP3A4 respectively,[3] as well as by several of Hypha's oxidising actinomycete strains. A functional microbial P450 was cloned from one of Hypha's strains and reproduced the human conversion of diclofenac to these hydroxylated products. The aim of the project was to improve whole-cell biotransformation efficiency of this microbial P450 cell factory to obtain drug metabolites.



Process summary

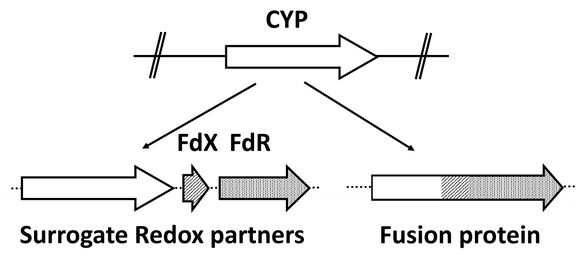
The P450 was cloned in an IPTG-inducible T7 expression plasmid by Circular Polymerase Extension Cloning. All other manipulations were performed using traditional molecular biology & Gibson Assembly. All recombinant *E. coli* BL21 (DE3) strains were cultured in a proprietary defined medium (PCM8.1), induced with IPTG and supplemented with 5-ALA and $FeSO_4$. Pellets were harvested by centrifugation and stored at -20 °C for resting cell biotransformation assays.

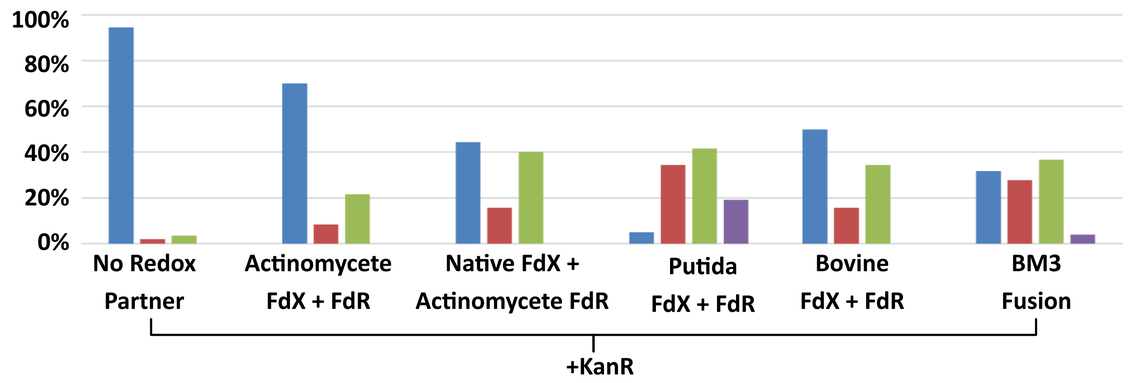
Resting cell biotransformation assays were performed at 27 °C, shaking at 300 rpm with resuspended cell pellets to an OD of 20 with 50 mM KPi, pH 7.4, 5 mM $MgCl_2$, 100 mM glucose and 0.1 mg/mL of drug. Reactions were stopped 24 hours later by quenching the reaction with 1:1 (v/v) acetonitrile and extracts were analysed by UPLC-ESI-QMS.

Results

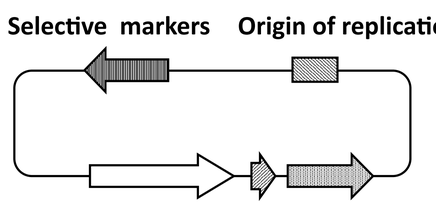
PolyCYP6 converts **diclofenac** (blue bar in graphs below) into the **4'-hydroxydiclofenac**, **5-hydroxydiclofenac** and **4',5-dihydroxydiclofenac** (red, green and purple bar respectively in graphs below). Endogenous redox partners present in *E. coli* BL21 (DE3) were compatible with PolyCYP6;[4] however, yields of the hydroxylated products were low.

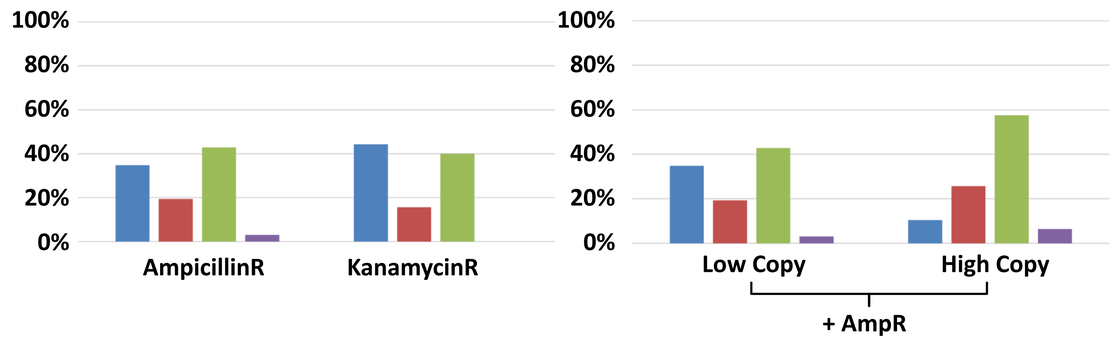
Diclofenac conversion to its hydroxylated products were improved through the co-expression of native or surrogate redox partners from actinomycete organisms,[5] *Pseudomonas putida* and *Bos taurus* or fusion with reductase domains of BM3 from *Bacillus megaterium.*[6] The best overall conversion detected was 95% with an operon containing the *P. putida* ferredoxin (FdX) and ferredoxin reducatase (FdR).





Modifications of vector components can also influence the expression of functional P450. Using the native FdX redox partner, 4',5-dihydroxydiclofenac was only produced with the ampicillin selection marker. Conversions to the hydroxylated products were further increased using a high copy vector.





Conclusions

- PolyCYP6 was successfully mined from one of Hypha's talented oxidising actinomycete strains and has demonstrated utility for hydroxylating compounds to generate human drug metabolites.
- Diclofenac conversions were greatly improved through the use of selected redox partners and modifications of components on the expression vector, a technique which allows for a more cost-effective and scalable method for production of drug metabolites in the milligram to gram quantity scale.
- Modular assembly of the expression vector allows optimisation of other functional Class I P450s mined from Hypha's strain collection to improve whole-cell biotransformation and catalytic activity efficiency.

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References

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Hypha Discovery Ltd is a UK-based CRO providing solutions to pharmaceutical and agrochemical R&D partners through the production of mammalian and microbial metabolites, as well as specialising in microbially-derived chemicals. We have delivered projects for 7 of the top 10 pharma companies and 4 out of 5 of the top agrochemical companies in provision of metabolites.