A hydroquinone specific screening system for directed P450 evolution

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Introduction

The direct hydroxylation of benzene to hydroquinone (HQ) under mild reaction conditions is not feasible by conventional organic chemistry. Recently, the heme dependent monooxygenase P450 BM3 has gained great interest regarding the hydroxylation of diverse benzenes [1]. An effective screening strategy is crucial for the identification of improved enzymes with desired characteristics in large mutant libraries. We established a first screening system that allows the quantification of dihydroxylated aromatic compounds obtained in P450-catalyzed reactions in a 96-well MTP format [2].

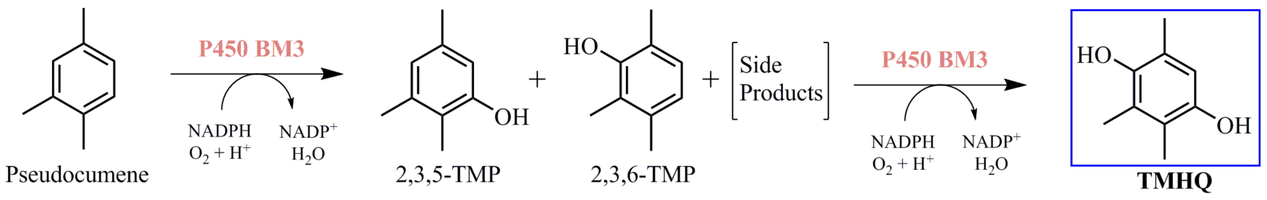

Fig. 1 Hydroxylation of pseudocumene by P450 BM3 to produce TMHQ.

- Hydroxylation of pseudocumene by P450 BM3 as target reaction for the establishment of the assay
- Pseudocumene hydroxylation by P450 BM3 leads to the formation of trimethylhydroquinone (TMHQ) (Fig.1) which is a key tocopherol precursor and of industrial relevance [3].

Assay Principle

- 4-Nitrophenylacetonitrile (NpCN) interacts with HQ under alkaline conditions [4] and forms a colorimetric detectable complex.
- Color formation is only obtained when at least two –OH groups are on the aromatic ring (Fig. 2).
- Depending on the substitutions of HQ, different maximal absorption and colors are obtained.

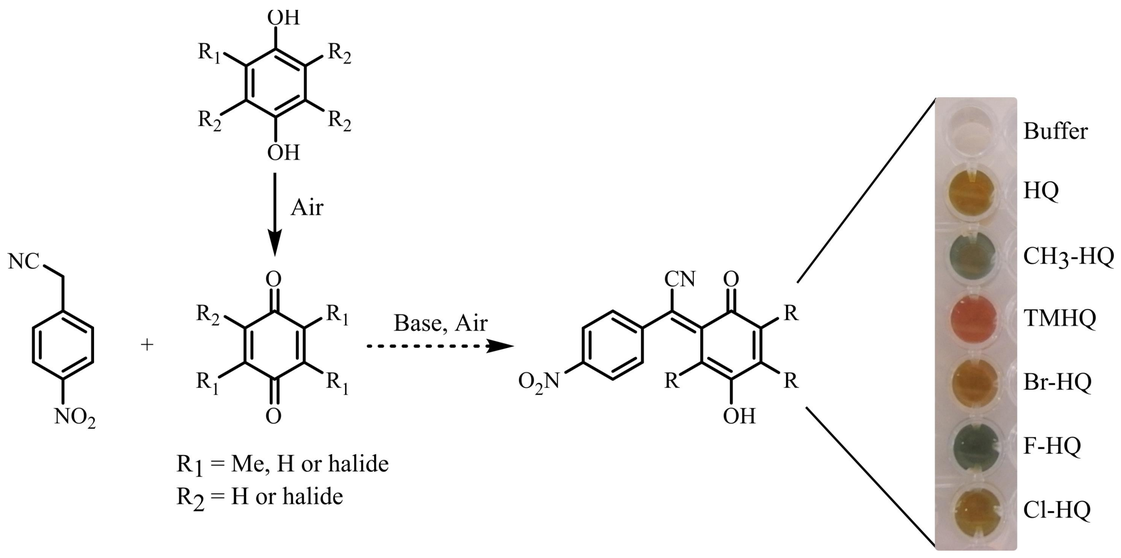

Fig. 2 Plausible product formation of NpCN with HQ. HQ concentrations were 50 µM in a total of 300 µL phosphate buffer. Color formation was obtained directly after adding 20 µL (0.04%) NpCN and 20 µL (2%) NaOH.

- Broad linear detection range and high sensitivity was obtained (1 to 250 µM TMHQ).

Screening strategy

- Combination with the widely used NADPH depletion assay possible [5].
- Glucose dehydrogenase (GDH) and glucose can be supplemented for NADPH regeneration to achieve longer conversion (Fig. 3).

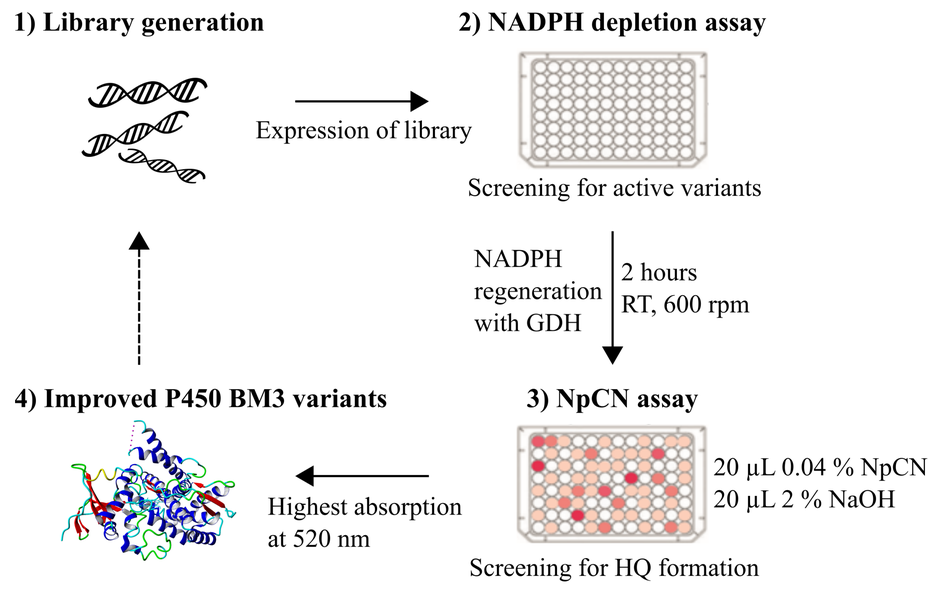

Fig. 3 Strategy for P450 evolution by applying the NpCN assay in combination with the NADPH depletion assay.

- Standard deviation of the NpCN assay in MTPs after 2 hours for detection of TMHQ formation was 14% using 93 repetitions of P450 BM3 M3 (R47S, Y51W, I401M, A330F) [3].

Validation – Screening for improved TMHQ formation

- A SSM library on position 330 using P450 BM3 AW1 (R47Q, Y51F, I401M) as starting variant was screened with the NpCN assay for improved TMHQ formation.

Table 1. TMHQ obtained in pseudocumene conversions by P450 BM3 WT and variants.

P450 BM3 variants	TMHQ [mM] after 2 h	TMHQ [mM] after 24 h
AW1	0.46 ± 0.01	1.11 ± 0.15
AW2	0.77 ± 0.06	2.16 ± 0.22
M3	0.49 ± 0.06	1.23 ± 0.09
WT	n.d.	0.02 ± 0.01

- P450 BM3 AW2 (AW1, A330P) was identified which showed the highest absorption values in the NpCN assay.
- A TMHQ formation up to 2.2 mM was obtained for P450 BM3 AW2 (Table 1).

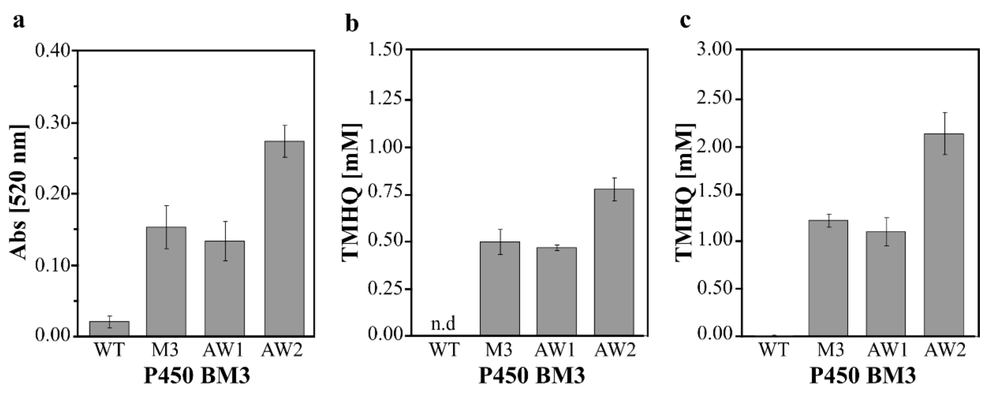

Fig. 4 TMHQ formation of different P450 BM3 variants detected with the NpCN assay and GC-FID. (a) NpCN assay; absorption at 520 nm. (b) TMHQ detected by GC-FID after 2 h and (c) 24 h.

- Absorption values obtained with the NpCN assay correlated well with the GC-FID results (Fig. 4).

Conclusion

A hydroquinone specific screening system was developed and validated by screening of SSM libraries, yielding P450 BM3 AW2 (R47Q, Y51F, A330P, I401M) with a TMHQ formation of up to 2.2 mM. The low detection limit (5 µM), the broad linear detection range (1 to 250 µM), and broad substrate scope (different aromatics) make the NpCN screening system broadly applicable for the detection of new enzyme variants.

References

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Acknowledgement

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