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Supplementary information

A Green Enzymatic Route for the Biotransformation of Naphthalene to Phthalic Acid

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1. Materials and Methods

1.1 Cloning and expression of Unspecific Peroxygenase (UPO)

The synthesis and cloning of AaeUPO (Jawa variant) gene into the pJRoC30 vector were carried out by Genewiz (Suzhou). The resulting recombinant plasmid pJRoC30-AaeUPO was transformed into *Saccharomyces cerevisiae* INVSc1. After incubation at 30°C for 4-5 days, single colonies were inoculated into liquid pre-culture (30°C, 220 rpm, 48 h). An equal volume of pre-culture was transferred to 100 mL of SC-U medium containing 25 μg/mL chloramphenicol and grown for 6-8 h (30°C, 220 rpm) until the OD_{600} reached approximately 1. The culture was subsequently transferred to 1000 mL of expression medium and incubated (25°C, 220 rpm) for 72 h until the OD_{600} reached 25-30. The AaeUPO protein was expressed and secreted into the supernatant. The crude enzyme solution was clarified using a hollow-fiber microfiltration device (0.45 μm membrane) and then concentrated to 10%-20% of its initial volume using a hollow-fiber ultrafiltration device (5 kDa molecular weight cutoff). The resulting concentrate was further clarified (0.22 μm filter) and refined using a 10 kDa centrifugal ultrafiltration tube, with a concurrent medium exchange for 20 mM potassium phosphate buffer (KPB, pH 7.0) via diafiltration. The purity and presence of the recombinant AaeUPO were validated through 12% SDS-PAGE analysis.

1.2 Expression and purification of 2,3-DHBD, 1-HNDO, PhdK, Nox and NsaE.

To construct the multi-enzyme cascade system for 1-naphthol transformation, the genes encoding 2,3-dihydroxybenzoate decarboxylase, 2,3-DHBD (P80402.2, from *Aspergillus oryzae RIB40*), 1-hydroxy-2-naphthoate dioxygenase, 1-HNDO (ABM11315.1, from *Mycolicibacterium vanbaalenii* PYR-1), 2-carboxybenzaldehyde dehydrogenase, PhdK (Q79EM7.1, from *Nocardioides* sp. KP7), NADH oxidase Nox (WP_050338260, from *Lactiplantibacillus pentosus*) and *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase, NsaE (NP_863078.1, from *Pseudomonas putida*) were cloned into the pRSFDuet1 vector. The plasmids were transformed into *E. coli* BL21 (DE3) for expression. Recombinant cells were cultivated in 1 L of LB broth and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at 20°C with shaking at 220 rpm for 24 h. Cells were harvested by centrifugation (5000 rpm, 30 min), resuspended in Buffer A (20 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) and lysed using a high-pressure cell disruptor set to 800 bar. Lysate was clarified by centrifugation (12000 rpm, 50 min), loaded onto a pre-equilibrated HisTrap FF Crude column (Cytiva) and washed with Buffer A. Protein was eluted by increasing the ratio of Buffer B (250 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) from 0 to 100% over 10 column volumes (CV). Fractions were analyzed by 12% SDS-PAGE, and those containing the target protein were pooled and concentrated using a centrifugal protein concentrator (Millipore, 10 kDa MWCO). Subsequently, the proteins were desalted into 100 mM potassium phosphate buffer (KPB, pH 7.0) using a HiPrep 26/10 Desalting column. Finally, the purified proteins were aliquoted, snap-frozen in liquid nitrogen, lyophilized (yielding approximately 20-40 mg of protein powder per liter of culture), and stored at -80°C until use.

1.3 Enzyme activity assays

AaeUPO crude enzyme activity was determined using an absorbance-based assay with 0.3 mM ABTS as the substrate. The reaction was performed in 100 mM sodium phosphate/citrate buffer (pH 4.4) with a final volume of 1 mL and initiated by the addition of 2 mM H_2O_2. This acidic pH was employed as the standard condition for the ABTS assay to maintain the stability of the generated ABTS radical cation. Absorbance was monitored at 418 nm using a spectrophotometer. The ABTS method was selected for its strong signal response and minimal interference from the culture medium color.

The activities of each of the enzymes added into the cascade were verified as follows: The activities of both 2,3-DHBD and 1-HNDO were determined via fluorometric detection. An increase in fluorescence indicates the carboxylase catalyzes the conversion of 1-naphthol to 1-hydroxy-2-naphthoic acid (1-H2NA); meanwhile, ring cleavage of 1-H2NA to 2′-carboxybenzyl-pyruvic acid by the 1-HNDO dioxygenase would result in a decrease in fluorescence. Both the activities of 2,3-DHBD and 1-HNDO were determined by monitoring fluorescence changes in a black microtiter plate (MTP) using the Tecan Infinite M200 Pro microtiter plate reader (Zurich, Switzerland) (λ_{Ex}=350 nm, λ_{Em}=420 nm). PhdK dehydrogenase activity was confirmed by an increase in absorbance at 340 nm (indicating NADH formation) during the oxidation of 2-carboxybenzaldehyde to phthalic acid; Nox oxidase activity was evidenced by a decrease in absorbance at 340 nm (indicating NAD^+ regeneration). The activity assay systems for AaeUPO, 2,3-DHBD, 1-HNDO, PhdK, and Nox are detailed in Supplementary Tables S1–S5, respectively.

Table S1 Assay system for AaeUPO activity

Reaction system	Working concentration (mM)
ABTS	0.3
H_2O_2	2
Citric acid–sodium citrate buffer (pH 4.4)	100

Table S2 Assay system for 2,3-DHBD activity

Reaction system	Working concentration (mM)
$KHCO_3$	200
1-naphthol	5
KPB buffer (pH 7.0)	100

Table S3 Assay system for 1-HNDO activity

Reaction system	Working concentration (mM)
1-Hydroxy-2-naphthoic acid	0.5
KPB buffer (pH 7.0)	100

Table S4 Assay system for Phdk activity

Reaction system	Working concentration (mM)
2-Carboxybenzaldehyde	2
NAD^+	2
KPB buffer (pH 7.0)	100

Table S5 Assay system for Nox activity

Reaction system	Working concentration (mM)
NADH	2
KPB buffer (pH 7.0)	100

1.4 Reaction conditions for the one-pot biotransformation of naphthalene into phthalic acid

The reaction was carried out at 30°C, with a reaction volume of 1 mL. 20% (v/v) acetonitrile was added as a crucial organic co-solvent to enhance naphthalene dispersion. The reaction system comprised 200 mM $KHCO_3$, 0.2 mM NAD^+, 100 mM KPB buffer (pH 7.0), and naphthalene (2 and 5 mM loadings) as the substrate. The integrated one-pot cascade reaction comprised 0.75 mg mL^{-1} each of 2,3-DHBD, 1-HNDO, PhdK, Nox, NsaE. 200 µL of concentrated AaeUPO enzyme was added into the system to initiate the cascade reaction. The entire reaction was maintained for 12 hours, after which the process was terminated, and the products were quantified via HPLC analysis.

1.5 HPLC analysis

Chromatographic separation was carried out using a Thermo Dionex UltiMate 3000 HPLC system with an UltiMate XB-C18 column (4.6 × 250 mm, 5 µm). The mobile phase, composed of methanol and water (with 0.1% phosphoric acid), was pumped at a constant flow rate of 1.0 mL min^{-1}. The gradient program was set as follows: a linear increase from 30% to 45% methanol (0–20 min), followed by 45% to 80% methanol (20–40 min), and finally re-equilibration to 30% methanol (40–45 min). UV absorbance was recorded at wavelengths of 275 nm and 300 nm.

2. Supporting Figures

2.1 Purification and characterization of 2,3-DHBD, 1-HNDO, PhdK, Nox and NsaE

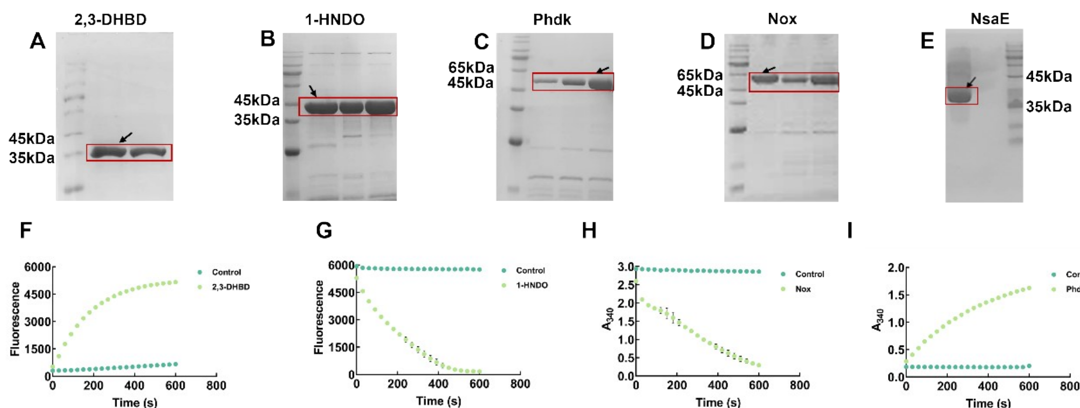
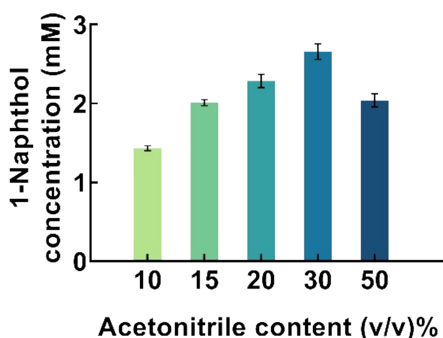


Figure S1 12% SDS-PAGE analysis and functional validation of purified enzymes. Purified proteins and their respective activities were characterized for (A, F) 2,3-DHBD carboxylase (38.9 kDa); (B, G) 1-HNDO dioxygenase (40.6 kDa); (C, I) PhdK dehydrogenase (51.8 kDa); (D, H) NADH oxidase (49.5 kDa); and (E) NsaE (36.9 kDa). Panels (A-E) represent the SDS-PAGE profiles confirming protein purity and molecular weight, while panels (F-I) show the corresponding activity assays (see Tables S2-S5 for details).

Note: A separate in vitro activity assay for NsaE was not performed, as its substrate (2'-carboxybenzylpyruvate) is a transient cascade intermediate that is difficult to isolate. The functional activity of NsaE is inherently validated in situ by the successful production of phthalic acid in the integrated cascade.



2.2 Effects of naphthalene loading and acetonitrile amount on 1-naphthol yield

Figure S2 Effect of acetonitrile (MeCN) content on the AaeUPO catalyzed hydroxylation of naphthalene at 10 mM loading. Increasing the MeCN content from 10 to 30% (v/v) effectively

solubilized naphthalene within the reaction system, enabling effective hydroxylation of naphthalene. Nonetheless, further increasing the MeCN content to 50% (v/v) results in a detrimental effect on AaeUPO activity, thereby reducing the yield of 1-naphthol.

2.3 Functional validation of the individual downstream modules: ring-opening and dehydrogenation reactions

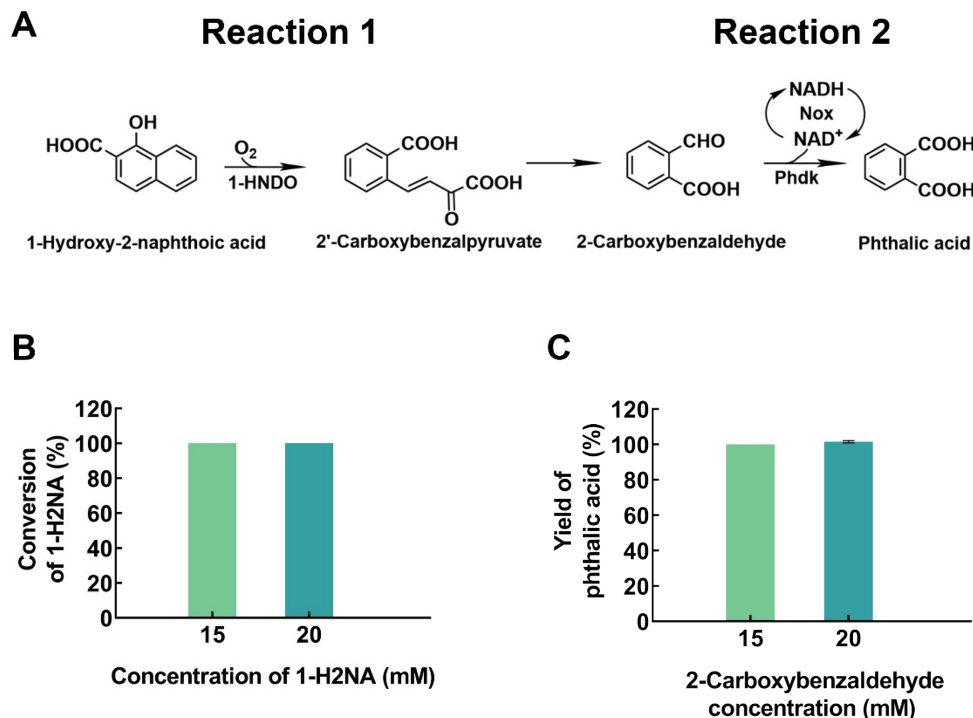


Figure S3 Functional validation of the individual downstream modules. (A) Schematic representation of the downstream reaction pathway of 1-naphthol conversion to phthalic acid. (B) Reaction 1: Ring-opening of 1-hydroxy-2-naphthoic acid (1-H2NA) catalyzed by 1-HNDO. Conversions of 1-H2NA at concentrations of 15 mM and 20 mM with 0.75 mg mL^{-1} 1-HNDO exceeded 99%. (C) Reaction 2: Dehydrogenation of 2-carboxybenzaldehyde to phthalic acid. Near-complete conversions (>99%) of 2-carboxybenzaldehyde at concentrations of 15 mM and 20 mM, catalyzed by 0.75 mg mL^{-1} PhdK and Nox. Both reactions were carried out at 30°C in 0.1 M potassium phosphate buffer, pH 7.0. Results were determined by HPLC analysis.