Enzyme Cascades for Synthesis of Value-Added Molecules from Carboxylic Acids

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To better incentivize the collection and recycling of plastic waste, new chemical transformations must be developed. Polyethylene terephthalate (PET) is a recalcitrant plastic whose deconstruction through chemical or biological means has recently received much attention. However, a limited number of functionalized products have been formed from aromatic deconstruction products like terephthalic acid (TPA) and mono(2-hydroxyethyl)terephthalate (MHET). Here, we explore using TPA and MHET from PET deconstruction as starting materials for the green synthesis of valuable aryl (di)amines through biocatalysis. Biocatalysis and microbial fermentation efforts in this space have long focused on production of short- and medium-chain aliphatic diamines. Even so, there is a distinct gap for synthesis of (di)amines with an aromatic moiety, many of which may prove useful in applications such as formation of nonisocyanate polyurethanes and polyamides.

The first part of this work focuses on developing a multienzyme cascade to synthesize (di)aldehydes and (di)amines from PET deconstruction products like TPA and MHET. Through a bioprospecting approach, we purified and characterized ATP- and NADPH-dependent carboxylic acid reductases (CAR), a class of enzymes that reduce carboxylic acids to aldehydes. We identified the CAR from *Segniliparus rotundus* (srCAR) as having both the broadest substrate scope and the highest activity toward TPA and MHET out of 17 tested enzymes. Building on this, we coupled aldehyde biosynthesis with enzymatic reductive amination in a one-pot reaction by applying the ω-transaminase from *Chromobacterium violaceum* (cvTA). After pairing the system with enzymatic cofactor regeneration, the final 5-enzyme reductive amination platform converted 10 mM TPA to *para*-xylylenediamine (pXYL) at 69% yield, and 5 mM MHET to *para*-aminomethylbenzoic acid (pAMBA) at 70% yield. By combining the synergies of chemical depolymerization with biocatalytic functionalization, we show, to our knowledge, the first reports of CAR specificity towards TPA and enzymatic production of TPAL and pXYL. This work lays the foundation for eventual valorization of waste PET to higher-value materials that can be made from pXYL.

Next, we explored scale-up and process intensification of the multienzyme diamine synthesis cascade by applying *E. coli* cell-free lysates as the biocatalyst source. While lysates are cheaper to produce than purified proteins, they contain endogenous enzymes that can divert reaction intermediates, especially aldehydes, to off-target products. Under lysate conditions, we observed up to 28% accumulation of alcohol products due to overreduction of aldehydes by endogenous *E. coli* enzymes. To mitigate this issue, we generated lysates from *E. coli* MG1655(DE3) RARE.Δ16, a strain engineered to minimize aldehyde overreduction, which restored cascade selectivity. Building on this improvement, we then alleviated bottlenecks associated with ATP regeneration, allowing us to achieve an 87% HPLC yield of pXYL from a

2.5 mmol synthesis (50 mL, 50 mM). We then isolated pXYL, which yielded 174 mg of product, representing an overall yield of 51%.

In the final stage, we focused on enzyme engineering to improve process tolerance, substrate scope, and thermostability of CAR. We used ancestral sequence reconstruction (ASR) to develop CAR variants with improved performance. Starting from a panel of extant CARs that are active on aliphatic, aromatic, heterocyclic, and polycyclic substrates, we applied ASR to generate 39 ancestral CAR variants, of which we tested 34. Several ASR CARs showed enhanced thermostability and activity compared to extant enzymes. Notably, some ASR-derived CARs efficiently converted sterically demanding ortho-substituted benzoic acids into their respective aldehydes, expanding access to valuable scaffolds in pharmaceutical synthesis like quinolines and coumarins.

This work advances the valorization of PET-derived monomers by developing enzyme cascades for the synthesis of aldehydes and diamines, integrating process intensification, and exploring enzyme engineering. We established a multienzyme platform coupling CAR- and TA-catalyzed transformations with cofactor regeneration to convert TPA and MHET into value-added products like pXYL and pAMBA. Transitioning to *E. coli* cell-free lysates enabled cost-effective scale-up, while using the aldehyde-stabilizing strain RARE.Δ16 mitigated off-target reduction and improved selectivity. Finally, ancestral sequence reconstruction yielded CAR variants with enhanced thermostability, activity, and substrate scope, including efficient reduction of sterically hindered ortho-substituted benzoic acids, expanding access to high-value aldehyde synthons.