STRUCTURAL AND FUNCTIONAL ANALYSIS OF NON-HEME IRON ENZYMES FOR SUSTAINABLE BIOHYDROCARBON PRODUCTION

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ABSTRACT

The search for sustainable alternatives to fossil fuel dependence has led to the consideration of biohydrocarbons as a promising option due to their similar properties to petroleum-derived counterparts. One strategy for biohydrocarbon production is the enzymatic decarboxylation of fatty acids, employing non-heme iron-dependent decarboxylases such as UndAs. This study focuses on the discovery and heterologous expression of UndA-type decarboxylases and their characterization. The enzymes' capacity to produce n-alkenes from fatty acids was evaluated through gas chromatography, varying factors such as pH, temperature, and ascorbic acid concentration. Crystallization assays were performed under distinct conditions, and the obtained crystals were submitted to diffraction analysis. Among the enzymes studied, UndA676 and UndA828 showed significant activity, positioning them as key candidates for further molecular investigation. Structural studies revealed the three-dimensional structures of Und057 and Und828, revealing significant structural. These initial results underscore the potential of UndA676 and UndA828 in biohydrocarbon production and contribute to biotechnological approaches aiming at the sustainable production of olefins.

Keywords: Decarboxylases. Metalloenzymes. UndA. Fatty acids. Biohydrocarbons.

1 INTRODUCTION

The search for sustainable alternatives to the dependence on fossil sources has led to the consideration of bio-hydrocarbons as a promising option due to their properties similar to those derived from petroleum. One strategy for producing bio-hydrocarbons is the enzymatic decarboxylation of fatty acids, a process in which non-heme iron-dependent decarboxylases, like the UndAs, play a crucial role [1]. These enzymes catalyze the transformation of dodecanoic acid into 1-undecene and CO_2, demonstrating the importance of iron metals in substrate activation and high-valence intermediate formation [1,2]. The specificity and efficiency of UndA in converting fatty acids into olefins point to its biotechnological potential in generating renewable energy alternatives, as well as substituents for petrochemical sector. According to this, the main goal of the present work is the discovery, heterologous production followed by purification novel decarboxylases type UndA. Moreover, we intend to understand the molecular specificities and chemo-regioschemistry of the enzymes to get insight into catalytic rate improvements

2 MATERIAL & METHODS

Heterologous Expression in *E. coli*

The genes in Table 1 were acquired in pET28a vectors for transformation and expression in *E. coli* BL21 (DE3), following established protocols. The process involves growing transformed clones in M9 medium, inducing expression with 0.25 mM IPTG [2].

Table 1: Selected UndA-like genes

Target gene code	Protein	Microorganism	% ID with PsUnd (PDB: 6P5Q)
IHGDCBMJ_39676	UI676	Metagenome – LNBR/CNPEM	81,87
SQF99828.1	US828	US8281 *Paucimonas lemoignei*	81,23
WP_097267769.1	UW769	UW769.1 *Caballeronia udeis*	51,38
IHGDCBMJ_44057	UI057	Metagenome – LNBR/CNPEM	27,06
IHGDCBMJ_61187	UI187	Metagenome – LNBR/CNPEM	23,02

Protein Purification

Utilizing the pET28a expression system, proteins fused to a 6His tag were purified via affinity chromatography on nickel-sepharose columns, followed by SDS-PAGE verification. Pure samples were further concentrated and subjected to molecular exclusion chromatography.

Decarboxylation Activity

The ability to produce n-alkenes from fatty acids (C8 to C16) was evaluated using gas chromatography. Activity assays were conducted under various conditions, including pH, temperature, ascorbic acid concentration, and metal ions, to assess their impact on the enzymatic activity of UndAs. The reactions were carried out for 17 hours and stopped by the addition of HCl.

GC-FID Analysis for Alkenes Determination

Alkenes present in the reaction mixtures were identified using gas chromatography with a flame ionization detector (GC-FID). The samples were prepared through centrifugation and subsequently analyzed using precise chromatographic settings.

Structural studies by crystallography and x-ray diffraction

Crystallization experiments were carried out through vapor diffusion under precisely controlled conditions. Various experimental factors, including pH and different precipitants, were empirically tested with the aid of commercial kits. X-ray diffraction data were gathered at cryogenic temperatures at Sirius-CNPEM, Brazil.

3 RESULTS & DISCUSSION

In this study, five iron-dependent enzymes, namely Und057, Und187, Und676, Und769, and Und828, were explored for their potential in heterologous expression, purification, and decarboxylation activity towards the production of 1-undecene. Among these, the enzymes UndA676 and UndA828 demonstrated significant activity, marking them as key candidates for further biohydrocarbon production investigations. The results underline the enhanced performance of these enzymes under slightly acidic conditions and at lower temperatures, which are critical for preserving their optimal ionization states and stability (Figure 1).

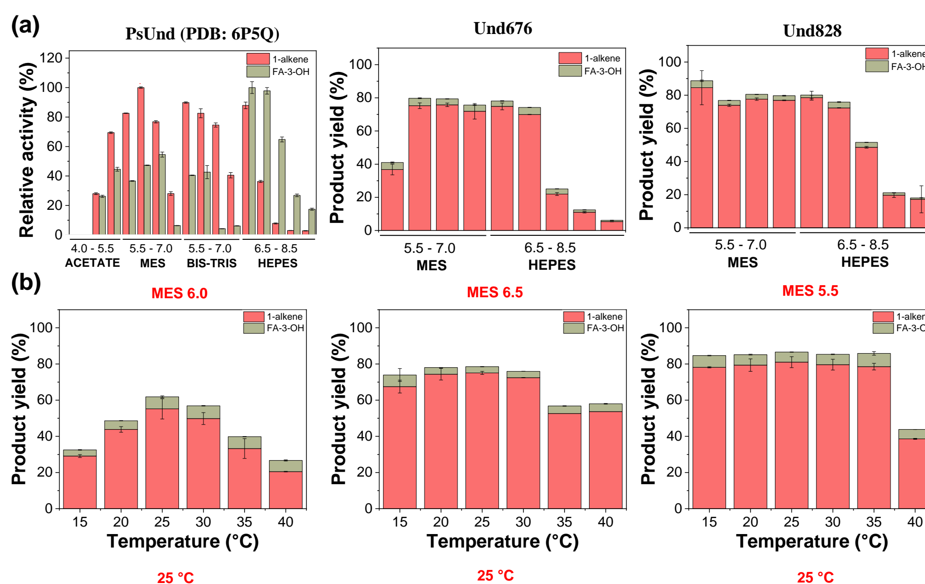

Figure 1 Assessment of decarboxylation activity of PsUnd, Und676, and Und828 under the influence of pH (a) and temperature (b). Conditions: PsUnd (150 µM, pH 6.0, 16h), Und676 (100 µM, pH 6.5, 17h), and Und828 (100 µM, pH 5.5, 17h), all with 500 µM C12 substrate, 10 mM ascorbate, and 850 rpm agitation.

Furthermore, the pivotal role of ascorbic acid as a reducing agent in this enzymatic process was identified, underscoring the necessity of fine-tuning experimental parameters such as pH, temperature, and reducing agent concentration to achieve maximum enzymatic activity. Ascorbic acid's function aligns with its known role in maintaining the iron cofactor in its reduced state, essential for the catalytic activity of iron-dependent enzymes [1].

The three-dimensional structures of the Und057 and Und828 enzymes were elucidated, achieving maximum resolutions of 2.8 Å and 2.0 Å, respectively, as shown in Figure 2. Despite their low primary sequence identity of 34.8%, a significant structural similarity in the main chain was noted, with an RMSD of Cα = 2.1 Å. Nonetheless, unique features in the catalytic site of the Und057 enzyme were identified, potentially accounting for its observed inactivity so far. Based on the structural data obtained, mutants have been designed to better understand the specificity, (regio) chemoselectivity and molecular drivers which are responsible for the decarboxylation reaction

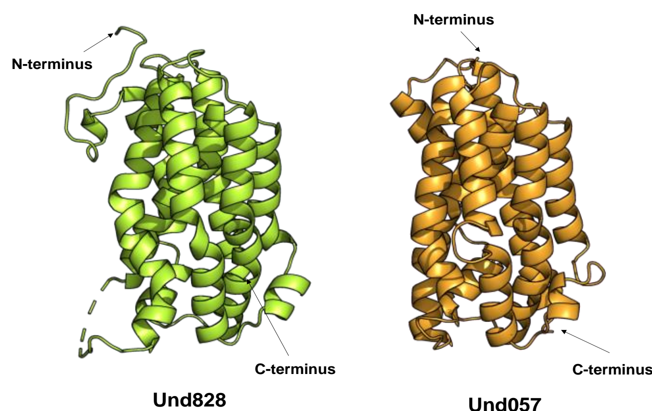

Figure 2 Three-dimensional representation of the Und828 and Und057 decarboxylases obtained at Sirius - CNPEM. The images were generated by the PyMol program.

4 CONCLUSION

The initial results obtained highlight the potential of UndA676 and UndA828 in biohydrocarbon production, as well as provide valuable insights into the enzymatic decarboxylation of fatty acids, an atypical reaction, by non-heme oxidative enzymes. Furthermore, the discovery and development of potential fatty acid decarboxylases significantly contribute to biotechnological approaches towards the sustainable production of olefins.

REFERENCES

[1] Zhang B, Rajakovich LJ, Van Cura D, Blaesi EJ, Mitchell AJ, Tysoe CR, et al. Substrate-Triggered Formation of a Peroxo-Fe2(III/III) Intermediate during Fatty Acid Decarboxylation by UndA. J Am Chem Soc. 2019;141(37):14510–4.
[2] Manley OM, Fan R, Guo Y, Makris TM. Oxidative Decarboxylase UndA Utilizes a Dinuclear Iron Cofactor. J Am Chem Soc. 2019;141(22):8684–8.

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