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Food Security, Sustainable Agriculture and Forestry, Marine, Maritime and Inland Water Research and the Bioeconomy

Call

H2020-FNR-2020: Food and Natural Resources

Topic name

FNR-16-2020: ENZYMES FOR MORE ENVIRONMENT-FRIENDLY CONSUMER PRODUCTS

FuturEnzyme:

Technologies of the Future for Low-Cost Enzymes for Environment-Friendly Products

Final ID: 101000327



AT LEAST 180 ENZYMES (RECOMBINANT,
NATIVE, BIOMIMETIC) WITH ATTRACTIVE
PROPERTIES, AVAILABLE

D4.7

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Document information sheet

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Summary

| | |
|--|----|
| At least 180 enzymes (recombinant, native, biomimetic) with attractive properties, available | 3 |
| 1. Scope of Deliverable | 3 |
| 2. Origin of the material | 3 |
| 3. Description of enzymes (recombinant, native, biomimetic) with attractive properties..... | 4 |
| 4. Methodology | 6 |
| 5. Enzymatic materials with properties attractive for the project | 8 |
| 7. Conclusions..... | 9 |
| 8. Annex..... | 10 |

At least 180 enzymes (recombinant, native, biomimetic) with attractive properties, available

1. Scope of Deliverable

This deliverable consists in a report detailing the identity of at least 180 enzymes (recombinant, native, biomimetic) that, after successful expression and testing, did show properties relevant to the project. The comparative analysis of all these enzymes and the corresponding datasets is key to further nominate the best target for genetic and supramolecular engineering (WP5), large scale production (WP6) and pre-industrial validations (WP7). The enzyme materials, the real-life substrates to be tested, and the information have been exchanged between partners, to guarantee that, although enzyme activities are initially assayed using in house methods and commercial substrates (close to those relevant to the project in some cases), they are subsequently assayed with real substrates relevant to the project. This information is associated with a QR code, which has been made available in the internal FuturEnzyme repository.

2. Origin of the material

As also detailed in deliverable D4.6 “The metadata on expression yield, activity and stability”, along the already 18 months of the project, different deliverables have been accomplished from which the present one nourishes. To be mentioned:

Deliverables in the frame of WP2:

- D2.2_Set of 250 000 sequences pre-selected (November 2021; to be updated)
In this deliverable, a set of about 3.2 sequences with interest for our project was retrieved by BLAST and HMM search, complemented with computational screens.
- D2.3_Set of 1000 enzymes selected using motif screens (May 2022; to be updated)
In this deliverable sequences retrieved in the frame of WP2 (deliverable D2.2) and WP3 (deliverable D3.3) were subjected to a filtering pipeline comprising the following criteria: confirmation of full-length sequence, presence and conservation of all proper domains and catalytic residues (along with MSA), the 3D structure modeling using AlphaFold 2.0, substrates (specified by the manufacturers) docking using Glide software (Schrödinger company) in the active site of the enzymes, and the substrate positioning around the active site with PELE (Protein Energy Landscape Exploration) software from BSC. The pipeline includes also the analysis of sequence coverage, the homology with reported similar sequences, the pair-wise similarity, the network analysis using the MCL (Markov Cluster Algorithm) algorithm. The idea of this pipeline was to select the priority enzymes to work with in WP4.
- D2.4_Set of 180 enzymes for experimental focus (July 2022; to be updated)
In this deliverable, at least 180 enzymes from the priority sequences retrieved in the frame of WP2 (deliverables D2.2, D2.3) and WP3 (deliverable D3.3), were preliminary selected to proceed with their cloning, synthesis, expression and characterization.

Deliverables in the frame of WP3:

- D3.3_Set of 100 clones, 10 isolates, 10 enzymes shortlisted for sequencing (March 2022; to be updated)
In this deliverable, bio-resources available before the beginning of the project and newly generated during the project were screened by naïve/functional methods to identify those with interest for our project. Bio-resources include previous and new enzymes, environmental samples, isolates, enrichments, and clone libraries that were checked for the purpose of the present project, and best selected ones sequenced and sequences with interest for our project were retrieved.

Deliverables in the frame of WP4:

- D4.2_The FuturEnzyme portfolio of 1000 enzyme (recombinant/native/biomimetic) material, obtained (September 2022)
In this deliverable, the expression, preparation and production of set of protein samples of about 1000 enzymatic materials were undertaken by members of the consortium in a variety of hosts (heterologous or

native) and vectors, cell-free systems, biomimetic metamorphosis systems, to cite some, as well as genetically-engineered mutants and supramolecular-engineered (immobilized) enzymes generated in the frame of WP5.

- D4.3_Cell-free expression reported system developed (September 2022)
In this deliverable, a cell-free expression system was developed that allow the production and detection of enzymatic activities in a high-throughput manner by skipping the step of recombinant expression.
- D4.4_Biomimetic protease production system, developed (September 2022, re-opened to be updated in month 30)
In this deliverable a green chemical-system was designed that allows the production of enzymes with inherent problems of expression, particularly, biomimetic proteases.
- D4.6_The metadata on expression yield, activity and stability available (November 2022)
This deliverable consists on the datasets informing about the expression yield, activity and stability of all enzymes generated in the project until month 18.

3. Description of enzymes (recombinant, native, biomimetic) with attractive properties

Deliverable D4.6 “The metadata on expression yield activity and stability” (November, 2022) compiled all the relevant information regarding all the 678 enzymatic materials from which datasets were generated until month 18. Based on the data reported in this deliverable, until month 18, 368 enzymatic materials (native enzymes, mutants, immobilized preparation, biomimetic, protein extracts, etc.) have been selected as candidates having characteristics of interest for the three applications relevant for the FuturEnzyme project: detergent, textiles and cosmetics. These 368 candidates are summarized and reported in this deliverable (D4.7), which are listed in **Table 1** (access provided in the Annex section). This table contains the ID name, the amino acid sequence, the GPS coordinates (if available), the physicochemical properties (denaturing temperature, optimal temperature and pH); the activity towards a variety of standard commercial substrates and real substrates relevant for the project are summarized. The screening method by which each of the enzymatic materials was retrieved is summarized in **Table 2**.

Table 2. Summary of methods by which each enzyme was retrieved.

| Screen method | Nr |
|---|-----|
| Homology screen | 244 |
| Functional screen | 78 |
| Homology and computational screen | 20 |
| Computational screen | 18 |
| Functional and homology (genome) screen | 5 |
| Biomimetic "metamorphosis" | 1 |
| Supramolecular engineering | 2 |
| TOTAL | 368 |

The enzymatic activity of each of the selected preparations is shown in **Table 3**, which confirms that the enzymatic materials with attractive properties cover all activities relevant to the project, with major representatives being lipases and hyaluronidases.

Table 3. Enzymes with attractive properties selected for closer inspection.

| Enzymatic activity | Nr. |
|---|-----|
| Esterase, lipase, polyethylene terephthalate (PET) degrading hydrolase | 333 |
| Hyaluronidase and other glycosyl hydrolases | 26 |
| PluriZyme and other artificial enzymes: 1 PluriZyme (esterase/protease) 1 PluriZyme (esterase/transaminase) 1 PluriZyme (esterase-xylanase) 2 Artificial degrading enzyme | 5 |
| Protease/peptidase | 1 |
| Biomimetic oxidase EH3-6-hexyl-[1,3,2]dioxaphosphepino[5,4-b:6,7-b']dipyridine 6-oxide | 1 |
| Immobilized lipase | 2 |

| | |
|--|------|
| TOTAL | 368 |
| Percentage (%) referred to total enzymes in D4.6 | 54.3 |

The nature of the enzymatic materials, selected as having interesting properties, is summarized in **Table 4**, and briefly described below:

- i) 355 enzymes which were assessed (after cloning or gene synthesis) for heterologous expression. All 355 enzymes with attractive properties can be produced (0.1 – 100 mg/L culture) as soluble proteins in *Escherichia coli* as host and using 10 different vectors (**Table 5**), as successfully proven in small- to medium-scale expression and purification culture volumes of 1-10 000 mL (See D4.2 “The FuturEnzyme portfolio of 1000 enzymes, recombinant, native, biomimetic, material”).
- ii) 10 enzymatic extracts from native hosts, whose enzymatic activities were confirmed by activity check. They include protein materials from a number of native hosts, including *Bacillus licheniformis*, *Bacillus subtilis subsp. Spizizenii*, *Bacillus vallismortis*, *Burkholderia multivorans*, *Citrobacter freundii*, *Haloarcula hispanica*, *Haloarcula salina HA08-SCL*, *Halobacterium salinarum*, *Haloferax mediterranei HA22-RCL*, *Halolactibacillus miurensis F4-4*, *Halorhabdus utahensis BNXHr*, *Halorhabdus utahensis SVXHr*, *Marinobacter sp.*, *Martellella sp.*, *Microbacterium oxydans*, *Nanohalococcus occultus SVXNc*, *Paenibacillus peoriae*, *Proteus mirabilis*, *Pseudomonas guineae*, *Pseudomonas protegens*, *Pseudomonas stutzeri*, *Psychrobacter celer*, *Serratia quinivorans*, *Stutzerimonas stutzeri*, and *Vibrio diabolicus*, to cite some.
- iii) 1 biomimetic with oxidase/protease activity.
- iv) 2 immobilized preparations with lipase activity.
Immobilized preparations (particularly those of Lip9 and Polur1 lipases), prepared at ca. 50-60 mg scale.

Table 4. Nature of the of the enzymatic material.

| Wild type, mutant, etc. | Nr |
|------------------------------|-----|
| Wild type enzymatic material | 343 |
| Mutants | 10 |
| Protein extracts or isolates | 12 |
| Biomimetic | 1 |
| Immobilized preparation | 2 |
| TOTAL | 368 |

Table 5. Summary of expression/production systems and expression/production level.

| Expression/production system | Nr |
|---|-----|
| <i>Escherichia coli</i> (pET-45b(+)) | 275 |
| <i>Escherichia coli</i> (pET22b) | 27 |
| <i>Escherichia coli</i> (Ek/LIC 46) | 23 |
| <i>Escherichia coli</i> (p15TV-L) | 16 |
| Native hosts | 12 |
| <i>Escherichia coli</i> (pBXNH3) | 3 |
| <i>Escherichia coli</i> (pET-46 Ek/LIC) | 3 |
| <i>Escherichia coli</i> (pQE306) | 2 |
| <i>Escherichia coli</i> De3 Lobstr(pMCSG53) | 2 |
| <i>Escherichia coli</i> (pBXCH) | 1 |
| <i>Escherichia coli</i> (pQE80L) | 1 |
| Biomimetic metamorphosis | 1 |
| Immobilization | 2 |
| TOTAL | 368 |

The enzymes, selected as having interesting properties, exhibited maximum amino acid sequence identities ranging from 100% to 36.9% to homologous proteins in public databases, with an average value (reported as %, with the interquartile range (IQR) in parentheses) of 87.9% (20.8%), and a pairwise amino acid sequence identity for all enzymes ranging from 11.9 to 99.7% (**Table 1**, access provided in the Annex section). Taken together, the primary sequence analysis of the suggests that the diversity of enzymes with attractive properties is not dominated by a particular type of protein or highly similar protein clusters, but consists of diverse non-redundant sequences assigned to multiple folds and subfamilies, which are distantly related to known homologues in many cases.

4. Methodology

The materials and methods established and used to test all enzymes materials produced in the frame of deliverables D4.2 “The FuturEnzyme Portfolio of 1000 enzymes (recombinant native biomimetic) material, obtained” and D4.6 “The metadata on expression yield activity and stability, available”, have been used for screen the enzymatic materials with most attractive properties relevant to the project. Although these materials and methods have been detailed in deliverable D4.6 “The metadata on expression yield, activity and stability, available”, they are described again for ease of access and readability.

Materials. Enzymatic materials were produced and expression yields determined using the host and strategies described in the deliverable D4.2 “The FuturEnzyme portfolio of 1000 enzyme (recombinant, native, biomimetic) material, obtained”.

Biochemical assays. Hydrolytic activity was determined, in multi-titer plates using appropriated spectrophotometers and other analytical techniques, by using multiple methods and substrates detailed in deliverable D3.2 “Standard assays, analytics and calculations for monitoring enzymatic performance”. In brief, these methods include:

- 2) Measuring the amount of *p*-nitrophenol released by catalytic hydrolysis of *p*-nitrophenyl (*p*-NP) esters and glycosides through modified methods reported by partners (see publications at the [Zenodo Community](#)). *p*-NP substrates include:
 - i) *p*-NP esters such as *p*-NP acetate, *p*-NP propionate, *p*-NP butyrate, *p*-NP hexanoate, *p*-NP octanoate, *p*-NP decanoate, *p*-NP dodecanoate, *p*-NP myristate and *p*-NP palmitate.
 - ii) *p*-NP sugars as *p*-NP L/D-galactopyranoside, *p*-NP beta-D-galactopyranoside, *p*-NP L/D-glucopyranoside, *p*-NP beta-D-glucopyranoside, *p*-NP L/D-mannopyranoside, *p*-NP L/D-maltopyranoside, *p*-NP beta-D-maltopyranoside, *p*-NP L/D-xylopyranoside, *p*-NP beta-D-xylopyranoside, *p*-NP L/D-glucuronide, *p*-NP beta-D-galactopyranoside tetra-acetate, *p*-NP L/D-ribofuranoside, *p*-NP N-acetyl-beta-D-glucosaminide, *p*-NP L-arabinopyranoside, *p*-NP beta-D-cellobiopyranoside, *p*-NP L-fucopyranoside, *p*-NP L-rhamnofuranoside, *p*-NP beta-D-arabinofuranoside, *p*-NP L/D-maltohexanoside, and *p*-NP N-acetyl-beta-D-galactosaminide.
- 3) Measuring the amount of acid released after the hydrolysis of short, medium and long-chain triglycerides, coconut oil, palm oil, and olive oil emulsified with gum arabic.
- 4) Measuring hydrolytic activity towards oil and fat stains from real-life stained swatches (**Figure 1**) outlined in D2.1 “Manufacturers’ needs and specifications: protocol”, using a high-throughput free fatty acid concentration measurements [NEFA kit](#). These substrates, relevant for detergent applications, included:
 - C-S-61, Beef fat, coloured with Sudan red on Cotton - 45 cm width
 - PC-09, Pigment with oil (below 60°C) on Polyester/Cotton - 90 cm width
 - C-S-05S, Mayonnaise with carbon black on Cotton - 90 cm width
 - C-S-10, Butterfat with colourant on Cotton - 45 cm width
 - P-S-16, Lipstick, pink on Polyester/Cotton - 45 cm width
 - C-S-17, Fluid make-up on Cotton - 90 cm width

- PC-S-132, High discriminative sebum BEY with pigment on Polyester/Cotton - 45 cm width.

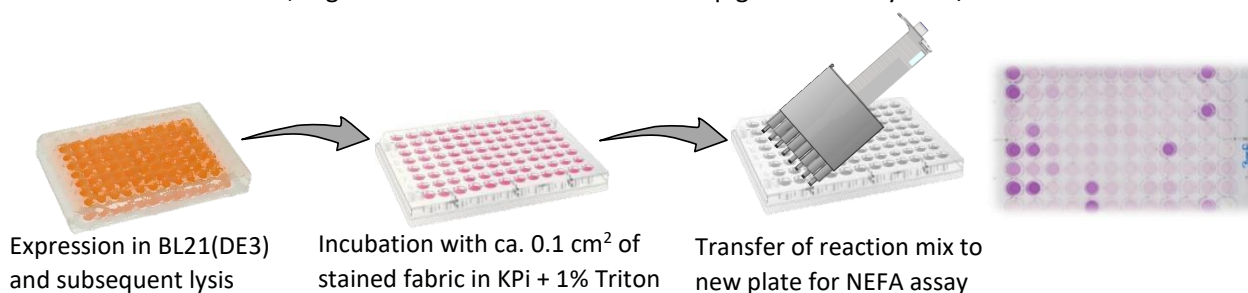


Figure 1. Workflow of assaying activity towards standard stained fabrics and assay plate showing active enzymes.

- Measuring the cleaning efficiency, as for real-life stained swatches (**Figure 1**), of residual spinning oils of synthetic materials (PES, PA and EL) / outlined in D2.1 “Manufacturers’ needs and specifications: protocol”, using a high-throughput free fatty acid concentration measurements [NEFA kit](#). These substrates, relevant for textile applications, included:
 - 61488, 61488Z ROH, 61488Z VORB, 92% PA, 8% EL 180g/m²
 - 61988, 61988F1 ROH, 61988F1 VORB, 92% PA, 8% EL 280g/m² – fatty acid esters, fatty acid amides, little silicone
 - 67007, 67007 ROH, 67007 VORB, 88% PA, 12% EL 135g/m² – mineral oil, paraffin, fatty acid ethoxylates, fatty acid amides, silicone
 - 3X58, 2X34G ROH, 3X58 VORB, 100% PES 100g/m² – fatty acid esters, mineral oil, paraffin
 - 66299, 5237/00 ROH, 92% CO, 8% EL 240g/m²
 - E03130, E03130 ROH, E03130 VORB, 80%PA6, 20%EL – fatty acid ethoxylates, fatty acid amides, silicone.
- Measuring the degradation of polymeric sugars and hyaluronic-based substrates outlined in D2.1 “Manufacturers’ needs and specifications: protocol”, using colorimetric and HPLC methods. These substrates, relevant for cosmetic applications, included:
 - α -cyclodextrin, β -cyclodextrin, γ -cyclodextrin, acarbose, pullulan, maltotriose, L-lactose, D-xylan, gum arabic, maltose, pectin, sucrose, trehalose, gellan gum, agarose, chitin, dextrin, dextran, amylose, starch, xanthan gum, hyaluronate, carboxymethyl cellulose, hyaluronic acid in MDa (Sigma-Aldrich)
 - Hyacare, High molecular weight (HMW, 700 kDa) hyaluronic acid produced after fermentation with *B. subtilis*
 - Hyacare50, Low molecular weight (LMW, 50 kDa) hyaluronic acid HyaCare® 50.
- Measuring the degradation of dyes and related substrates outlined in D2.1 “Manufacturers’ needs and specifications: protocol”, using colorimetric methods. These substrates, relevant for textile applications, included:
 - ABTS, nitrophenyldiamine and sinapic acid
 - Real-life dye BEMAPLEX Black D-HF
- Measuring the degradation of polyesters and plastics outlined in D2.1 “Manufacturers’ needs and specifications: protocol”, using colorimetric (i.e., turbidity decrease) and HPLC-UV/Vis analysis methods. These substrates, relevant for textile applications, included:
 - MHET (mono(2-hydroxyethyl) terephthalic acid), BHET (Bis(2-Hydroxyethyl) terephthalic acid), PET (polyethylene terephthalate), pre-treated amorphous PET foil, milled amorphous PET (UDUS) and pre-treated PET fabric, PET nanoparticles, Impranil DLN, polymeric polyesteramide and polylactic acid
 - Real-life pre-treated PET fabrics (Schoeller).

Determination of enzyme stabilities. To investigate stability and activities, the protocols detailed in deliverable D3.2 “Standard assays, analytics and calculations for monitoring enzymatic performance” were applied. The denaturation temperatures were determined by circular dichroism (CD) or nano-differential scanning fluorimetry (nanoDSF). CD spectra were acquired between 190 and 270 nm with a Jasco J-720 spectropolarimeter equipped with a Peltier temperature controller in a 0.1-mm cell at 25°C. The spectra were analyzed, and melting temperature (T_m) values were determined at 220 nm between 10 and 85°C at a rate

of 30°C per hour at appropriated concentrations and buffers that ensure protein stability. T_m , and the standard deviation of the linear fit, was calculated by fitting the ellipticity (mdeg) at 220 nm at each of the different temperatures. Protein melting curves measured by nanoDSF were determined using a Prometheus device (NanoTemper Technologies, Inc.), according to the manufacturer's recommendation, with the purified enzymes at protein concentrations of 4-8 mg/mL. Detergent stability was assessed by incubating and assaying enzymatic materials in washing liquor prepared according to D2.1 "Manufacturers' needs and specifications: protocol" using model esterase substrates. Alternatively, enzymes were incubated with laboratory-standard detergents like SDS. Stability towards water-miscible organic solvents was determined as described in Bollinger *et al.* ([Appl Environ Microbiol. 2020;86\(9\):e00106-20](#)).

5. Enzymatic materials with properties attractive for the project

In this deliverable, enzymatic materials with properties attractive to the project refer, at least, to the following cases:

- Those enzymatic materials (native enzymes, mutants, immobilized preparations, biomimetic scaffolds, protein extracts from native host, etc.) that show the capacity to degrade a variety of esters and oils in emulsions, stained swatches and spinning oils or dyes, or to hydrolyze hyaluronic acid or polyester fibers. Those constitute the basis to further nominate the at least 18 enzymes for WP5 (genetic and supramolecular engineering) (Deliverable 5.1), WP6 (large scale production) and WP7 (pre-industrial validations).
- Those enzymes that allow us to deepen into our understanding of enzyme performances and structure-function relationships, as well as to deepen into the possibilities to build sequence-based tools, including machine learning and Hidden Markov model (HMM) tools, for bioprospecting enzymes; these includes enzymes to validate predictive tools.
- Those enzymes that allow us to deepen into the possibilities to design artificial enzymes, namely, PluriZymes with different activities, or artificial enzymes capable of degrading PET polymers.
- Those enzymes that allow us to deepen into the possibilities to design a so-called biomimetic scaffold, namely, an enzyme scaffold into which a catalytic chemical entity is introduced.
- Those enzymes that allow us to deepen into the potential to evaluate the effect of different supramolecular engineering strategies to improve enzyme performances and stabilities.
- Those enzymes that constitute a proof of concept of cell free expression systems to further identify enzymatic activities of interest.
- Those isolates or protein extracts derived from them that after positive evaluation were selected for genome sequencing; pending the results of sequencing and synthesis of the genes of interest, they have been tested to find suitable culture conditions for the transformation of interest, e.g. cultures in different conditions in the presence of hyaluronic acid.

As detailed in the **Table 1** (access provided in the Annex section), for each of the three sectors targeted, the following number of candidates has been selected as having attractive properties:

- A set of sugar-transforming enzymatic preparations did show properties relevant for cosmetics applications (i.e., degradation of sugars, polymeric sugars and hyaluronic acid). Breaking down high molecular weight (HMW) hyaluronic acid to the desired size is the addressed application regarding the cosmetics relevant to the project, and we found 17 enzymatic materials (hyaluronidase-containing) being capable of degrading HMW hyaluronic acid as determined by colorimetric and chromatographic methods.
- A set of 333 (see **Table 2**, category "esterase, lipase, polyethylene terephthalate (PET) degrading hydrolases") of the enzymatic preparations support hydrolysis and degradation of a variety of esters, oils in emulsions, spinning oils in raw fabrics or stained swatch, dyes and related compounds, and/or polyethylene terephthalate (PET)-like fabrics, and thus they show properties relevant for both detergent and/or textile applications. Below are listed some relevant applications/purposes for which they were selected.
 - A set of 15 enzymes, 14 being native and 1 being a single mutant, out of the 333 candidates did show attractive properties for end-of-life textiles recycling (see **Table 1** (A) "degradation tests

with PET”) (see example at DOI 10.1101/2022.10.14.512230v1 in [Zenodo Community](#)). Two of them were selected as attractive candidates to validate cell-free expression systems (see example at DOI [10.1038/s41598-022-22383-x](#) in [Zenodo Community](#)).

- A set of 27 out of 333 enzymatic preparations have been identified with activity towards at least one standardized stained swatch, and 19 to hydrolyze pure oils in emulsions.
- A set of 260 out of 333 candidates detailed in **Table 1** (B) were selected as being attractive candidates to evaluate whether the enzyme thermal selection explains the plasticity of microbiomes in response to temperature, and thus to explain the adaptation of microbes to climate change. For that, temperatures for optimal temperatures, denaturing temperatures, and structural rigidity and flexibility have been examined and correlated with environmental parameters. The datasets have been found key to screen for enzymes with different thermal characteristics.
- A set of 10 out of 333 candidates (WP_101198885.1, WP_069226497.1, AJP48854.1, WP_042877612.1, WP_059541090.1, ART39858.1, WP_089515094.1, WP_026140314.1, PHR82761.1, and WP_014900537.1) were selected as being attractive candidates to deepen into the possibilities to build sequence-based machine learning tool for bioprospecting enzymes with specific properties (see publication at the DOI: 10.1002/anie.202207344 in [Zenodo Community](#)).
- A set of 14 out of the 333 candidates (EstLip_EH7, EstLip_IS12, EstLip_IS10, EstLip_IS11, EstLip_EH11, EstLip_EH15, EstLip_EH20, EstLip_EH22, EstLip_EH29, EstLip_EH30, EstLip_EH45, EstLip_EH59, EstLip_EH73, PET46) have shown attractive structures to deepen into our understanding on enzyme function (see example at DOI 10.1101/2022.10.14.512230v1, 10.1101/2022.09.10.507389v1, 10.1101/2022.09.17.508236v2, 10.3389/fmicb.2022.868839, 10.1111/febs.16554 in [Zenodo Community](#)).
- Two out of the 333 candidates, Lip9 and Polur1, are capable of degrading spinning oils in raw fabrics or stained swatch, and they were selected as initial target to evaluate the effect of different supramolecular engineering strategies to improve enzyme performances and stabilities (for details see deliverable D5.1).
- A set of 5 protein scaffolds (EH_{1AB1C}, FraC_{m1}, FraC_{m2}, TR_{2E2} and X11_mut1) were found as having attractive scaffolds for designing PluriZymes and artificial enzymes with interesting properties, namely scaffolds that combine transaminase and esterase activities, protease and esterase activities, xylanase and esterase activity, and also incorporating polyester degrading activity into a non-catalytic protein (see as example publications at the DOI: 10.3390/biom12101529 and 10.3390/ijms232113337 in [Zenodo Community](#)).
- One protein scaffold (EH₃) was selected as best candidates for the production and testing of biomimetic with oxidase activity, because the availability of structural data and wide active site.

The melting point/denaturing temperatures, optimal temperatures and pH, substrates being converted by each of the enzymatic materials, and the possible applications for each of them are extensively summarized in deliverable D4.6 “The metadata on expression yield activity and stability” (November 2022) and D5.1 “The shortlist of at least 18 enzymes nominated for engineering” (November 2022), and this is why they are not described again here.

7. Conclusions

As detailed in the Deliverable D4.6 “The metadata on expression yield activity and stability” (November 2022), a total of 678 enzymatic materials were successfully generated and produced until month 18. The comprehensive analysis of their datasets, which have been shared within the consortium (deliverable D4.6), revealed a sub-set of 368 enzymatic materials (native enzymes, mutants, immobilized preparation, biomimetic, protein extracts, etc.) with properties attractive for different purposes. Both the identities and corresponding datasets for each of these enzymatic materials have been communicated to all partners and the corresponding materials are available upon request by partners. For details see **Table 1** (access provided in the Annex section).

8. Annex

Table 1. Detailed information on expression, activity and stability of the 368 enzymes with properties attractive for the project. Shown are, among other datasets: 1) Enzymatic activity; 2) Name of the candidate; 3) Screen method; 4) Expression host; 5) Expression level; 6) Amino acid sequence or genome sequencing status; 7) Origin; 8) Details of stability features including denaturing temperature (Td), detergent stability; 9) Details of activity features, including substrate profile, optimal temperature and pH, etc.; 10) Sequence homology. Document available *D4.7_Annex 1_At least 180 enzymes with attractive properties_FINAL* at the FuturEnzyme web intranet through the following QR code (password needed), in the section *Shared data, Datasets*:

