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

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# SET OF HIGH-PERFORMING MULTI- ENZYME BLENDS

D4.8

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## Document information sheet

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## Set of high-performing multi-enzyme blends

### 1. Scope of Deliverable

This deliverable consists in materials representing multi-enzyme blends for formulating detergents and textiles. This deliverable is accompanied by a report detailing the enzymes used to prepare each of the multi-enzyme blends and their concentrations. This information is associated with a QR code, which has been made available in the internal FuturEnzyme repository.

### 2. Origin of the material

One deliverable has been accomplished from which the present one nourishes: D5.1\_ The shortlist of at least 18 enzymes nominated for engineering (November 2022). In this deliverable, the enzymes nominated as priority for the target applications are listed and the reasons for their priority detailed.

### 3. Description of nominated enzymes for multi-enzyme tests

Based on the information provided in D5.1, the following enzymes have been selected for multi-enzyme tests:

- The following enzymes have been selected for the production of cocktails to be used as additive in the Henkel liquid detergent: FE\_Lip9, FE\_ID9 and FE\_Polur1 (from CSIC), Kest3 and GEN0105 (Bangor), and EstLip\_Dim\_#008, EstLip\_TBec304, PEH\_Paes\_PE-H, PEH\_Pbau\_PE-H, and PEH\_Poce\_PE-H (UDUS), because they are capable to degrade the stained fabrics tested.
- The following enzymes have been selected for the production of cocktails to be used for the degradation of spinning oils from Schoeller fabrics: FE\_Lip9 (CSIC) and GEN0105 (Bangor).
- The following enzymes have been selected for the end-of-life fabric recycling of Schoeller PET-based fabrics: FE\_Lip9 (CSIC), PEH\_Paes\_PE-H, PEH\_Paes\_PE-H\_Y250S, PEH\_Pbau\_PE-H, PEH\_Pform\_PE-H, PEH\_Poce\_PE-H (UDUS), and GEN0105 (Bangor).
- The following enzymes have been selected for the production of cocktails to be used for the degradation of Schoeller dye: peroxidase I8AMQ8 and laccase Sav1970 (Bangor).

A combination of some of the best performing lipases for the degradation of stained fabrics, and thus to be used as additive in the Henkel liquid detergent, were tested. They include FE\_Lip9, FE\_ID9, Kest3 and GEN0105, for the reasons briefly summarized below (see D5.1 for details).

Name: FE\_Lip9

Activity: Lipase

Partner: CSIC, BSC

Source: MarRef - Marine Metagenomics Database

Expression system: *Escherichia coli* (pET-45b(+))

Expression level: Soluble (expression +)

Sequence (signal peptide underlined):

MKVMFVKKRSLQILIALALVIGSMAFIQPKEVKAAEHNPNVVMVHGIGGASYNFFSIKSYLATQGWDRNQLYAIDFIDKTGN  
NRNNGPRLSRFVKDVLDKTGAKKVDIVAHSMSGGANTLYYIKNLDGGDKIENVVTIGGANGLVSSRALPGTDPNQKILYTSV  
YSSADLIVVNSLSRLIGARNVLIHGVGHIGLLTSSQVKGYIKEGLNGGGQNTN

Synthesized sequence once introduced in the vector pET-45b(+):

MAHHHHHHVGTGSNDDDDKSPDPMAEHNPNVVMVHGIGGASYNFFSIKSYLATQGWDRNQLYAIDFIDKTGNNRNNGP  
RLSRFVKDVLDKTGAKKVDIVAHSMSGGANTLYYIKNLDGGDKIENVVTIGGANGLVSSRALPGTDPNQKILYTSVYSSADLIV  
VNSLSRLIGARNVLIHGVGHIGLLTSSQVKGYIKEGLNGGGQNTN

Nagoya protocol compliance: data recorded and shared with partners.

Biochemical features:

- Most active at temperatures close to 25-40°C, in agreement with its Td (41.70 ± 1.29°C)
- FE\_Lip9 retains more than 80% of its maximal activity at pH from 7.0 to 10.0.
- It also showed a half life time of 3.5 h in washing liquor.
- FE\_Lip9 was selected based on activity with short to large triglycerides.

- FE\_Lip9 is also capable to degrade all the stained fabrics tested (Pigment with oil on polyester/cotton (PC-09), Mayonnaise on cotton (C-S-05S), Lipstick, pink on polyester/cotton (P-S-16), Fluid make-up on cotton (C-S-17), High discriminative sebum BEY on polyester/cotton (PC-S-132), Beef fat on cotton (C-S-61) and Butterfat on cotton (C-S-10)), showing a preference for Butterfat on cotton (C-S-10).
- We also performed a free fatty acids assay with Schoeller's textiles where FE\_Lip9 showed a broad capacity to clean the oils in all raw textiles (61488F1, 3X58, 67007, 61988F1, 5237-00 and E03130).
- FE\_Lip9 do show PETase and BHETase activities, being capable of degrading PET.
- The lipase has been successfully subjected to supramolecular engineering (FHNW).
- The enzyme was successfully produced at 10 L scale: 3.96 grams of pure protein obtained.

Name: FE\_ID9

Activity: Lipase

Partner: CSIC

Source: Microbial assemblages from bone surface and the bone-eating worm *Osedax mucofloris* (BioProject ID PRJNA606180), Byfjorden, Bergen, Norway (60.397093N; 5.301293E) (collection date: from 01.2017 to 11.12.2017).

Expression system: *E. coli* (pET-45b(+))

Expression level: Soluble (expression +)

Sequence (no signal peptide):

MTNLSKPIPNPREYPILPPDMNYIYFENAHLFPFEPEKRDYSPVNAWWLSECAFLVYCHPGFARMAMALVGFDFHFFQGGKTECMVSWNKDSIIVAFRGTEMKLSAFHELRTDLNTAPVDFDKGSKVHKGFLKGLQEIWEGEEGLKLFLETLSAEAPSRSMWICGHS LGGALAALCFARLEKASGLYIYGAPRIGDGEFVRICDNRPVWRVEHGRDPIPLVPPDVPALNFNFKDMGKLIYIDYRGEILFERPLVTVEEEKSKVLLNISQQRKRRESLSVEGFKGVLDKDRAKTLINGINEHIMQSRVEWKEYFDSLDKGIGLKIKDHMPYIYCAKLWNILIEGL

Synthesized sequence once introduced in the expression vector:

MAHHHHHHVGTGSND DDDKSPDPMNLSKPIPNPREYPILPPDMNYIYFENAHLFPFEPEKRDYSPVNAWWLSECAFLVYCHPGFARMAMALVGFDFHFFQGGKTECMVSWNKDSIIVAFRGTEMKLSAFHELRTDLNTAPVDFDKGSKVHKGFLKGLQEIWEGEEGLKLFLETLSAEAPSRSMWICGHS LGGALAALCFARLEKASGLYIYGAPRIGDGEFVRICDNRPVWRVEHGRDPIPLVPPDVPALNFNFKDMGKLIYIDYRGEILFERPLVTVEEEKSKVLLNISQQRKRRESLSVEGFKGVLDKDRAKTLINGINEHIMQSRVEWKEYFDSLDKGIGLKIKDHMPYIYCAKLWNILIEGL

Nagoya protocol compliance: data recorded and shared with partners.

Biochemical features:

- Remarkable high activity at 40°C and pH 9.5.
- Capacity to degrade all the stained fabrics tested (Pigment with oil on polyester/cotton (PC-09), Mayonnaise on cotton (C-S-05S), Lipstick, pink on polyester/cotton (P-S-16), Fluid make-up on cotton (C-S-17), High discriminative sebum BEY on polyester/cotton (PC-S-132), Beef fat on cotton (C-S-61) and Butterfat on cotton (C-S-10)), showing a preference for Butterfat on cotton (C-S-10).
- Stable in the presence of washing liquor.
- The enzyme was successfully produced at 10 L scale: 10.2 grams of pure protein obtained.

Name: Kest3

Activity: Lipase

Partner: Bangor

Source: Abano Terme, Italy (45.362 N 11.789 E; isolated strain *Fervidobacterium pennivorans* DSM 9078; genome accession number CP003260.1)

Expression system: *E. coli* De3 Lobstr (p15TVL)

Expression level: Soluble (expression +)

Sequence (no signal peptide):

MDEKRSVKFFNKLTHIAKIILFTKGILILSSFFLAFSNFLLFTV VVILNVPLLRKSIFGRLPTDTKELRKS NVLSNQETYEYLPGLFLDVFYPSFFTESKKSQSVKGVILFAHGGGWISGYRRQPNNSWYRYLVSKGFIVATIDYERGYKAGIEK LIEELLQAVVFLNHLSSKLGINEKVS LMGSLAGGHLALLAASRIPERVKNVVAYYSPCDLLDIWHSASIFARFAAATTLKRLPTRARDVYERYSPINNITENYPTLLVHGLKDSVVPYFSSVKMFKTLREKGLAAKLLLHPKGDHGFVLRDRRTVDIIEKTAQFLEGKLW

Synthesized sequence:

MDEKRSVKFFNKLTHIAKIILFTKGILILSSFFLAFSNFLLFLT VVVILNVPLLRKSIFGRLPTDTKELRKS NVLSNQETYEYLPGLFL  
DVFYPSFFTESKKSQSVKGVILFAHGGGWISGYRRQPNNSWYRYLVSKGFIVATIDYERGYKAGIEK LIEELLQAVVFL ENHL  
SSKLGINEKVSMLGSLAGGHLALLAASRIPERVKNVVAYYSPCDLLDIWHSASIFARFAAATTLKRLP TRARDVYERYSPINNIT  
ENYPPTLLVHGLKDSVVPYFSSVKMFKTLREKGLAAKLLLHPKGDHGFVLRDRRTVDII EKTAQFLEGK L W

Nagoya protocol compliance: data recorded and shared with partners.

Biochemical features:

- Kest3 was selected based on activity with coconut oil.
- Topt 70°C (no significant activity reduction at temperatures up to 85°C). It showed comparable activity to commercial lipase from *Candida rugosa* (L1754, Sigma)).
- In the stained swatches tests Kest3 showed activity with Butterfat on cotton (C-S-10), Fluid make-up on cotton (C-S-17), Pigment with oil on polyester/cotton (PC-09), in the not buffered washing liquor.
- Though Kest3 showed a rate decrease in the presence of detergent, the Km decreased too leading to the catalytic efficiency increase 2.2 times (4.1 to 9.3 s<sup>-1</sup>\*mM<sup>-1</sup>).

Name: GEN0105

Activity: Lipase, PETase

Partner: Bangor

Source: metagenome of a mesophilic anaerobic digester, Evry, France (48.626 N 2.464 E)

Expression system: *E. coli* De3 Lobstr (p15TVL)

Expression level: Soluble (expression +)

Sequence (no signal peptide):

MSETSSASALPAYARIVVDKRAPFIRAILYLILRYVIKRS MKPDADILKLRAMQLRADQKYAHPAADAVMTPVDCDGVKAN  
WITLPGARPVIFYLHGGAWMFNFPRTYAAMLGRWARLLNARVLMVDYRLAPEHRYPAGANDCETAYRWLLAQGIDS  
KQIVIGGDSAGGNLTLTLLRLKSANQLPACAVALS PFVDFTLSSPMITNEKIDPMFTLEAMLGLRPHYLD PQDFLNVDAS  
PIFGDFSGLPPIFFQSSNTEMLRDESVRAAARAHQHGV TVELELWQHLP HVFQALQKLPQADAALQSIVRFINSHTGWQA

Synthesized sequence once introduced in the expression vector:

MSETSSASALPAYARIVVDKRAPFIRAILYLILRYVIKRS MKPDADILKLRAMQLRADQKYAHPAADAVMTPVDCDGVKAN  
WITLPGARPVIFYLHGGAWMFNFPRTYAAMLGRWARLLNARVLMVDYRLAPEHRYPAGANDCETAYRWLLAQGIDS  
KQIVIGGDSAGGNLTLTLLRLKSANQLPACAVALS PFVDFTLSSPMITNEKIDPMFTLEAMLGLRPHYLD PQDFLNVDAS  
PIFGDFSGLPPIFFQSSNTEMLRDESVRAAARAHQHGV TVELELWQHLP HVFQALQKLPQADAALQSIVRFINSHTGWQA

Nagoya protocol compliance: data recorded and shared with partners.

Biochemical features:

- GEN0105 was selected based on the outstanding activity with ester bond plastic (3PET, PLA, PCL). The enzyme was not found to be thermostable (data not shown).
- It was tested along with Kest3 on fatty standard stains (lipase activity present when screened on coconut oil). GEN0105 shows activity with Lipstick, pink on polyester/cotton (P-S-16), Butterfat on cotton (C-S-10), Fluid make-up on cotton (C-S-17), Pigment with oil on polyester/cotton (PC-09).
- Activities were comparable with Henkel enzyme mix, in the buffered washing liquor, though losing activity significant in the not buffered conditions.
- Good candidate for increasing stability.

#### 4. Experimental set-up for multi-enzyme tests

**Materials.** His<sub>6</sub>-tag enzymes FE\_Lip9 and FE\_ID9 available in the vector pET-45b(+) (supporting isopropyl β-D-1-thiogalactopyranoside-induced expression) and the host *E. coli* BL21(DE3). His<sub>6</sub>-tag enzymes Kest3 and GEN0105 available in the vector p15TVL (supporting isopropyl β-D-1-thiogalactopyranoside-induced expression) and the host *E. coli* De3 Lobstr. Luria-Bertani (LB) broth agar and LB broth (ref. 22700-041, Invitrogen), ampicillin (CAS nr. 69-53-4), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (CAS nr. 7365-45-9; ref. BP310-5, Fisher Bioreagents), isopropyl β-D-1-thiogalactopyranoside (IPTG) (CAS nr. 367-93-1), and Lysonase Bioprocessing reagent (Novagen, Darmstadt, Germany) were used. Ni-NTA His-Bind resin (from Merck Life Science S.L.U., Madrid, Spain). Glass microfibre filter 47 mm diameter (ref. MFV/1047, MFV1, Filter-LAB).

**Buffer.** The washing buffer (50 mM sodium phosphate buffer pH 8.0, 0.3 M NaCl and 10 mM imidazole) was prepared by mixing 13.25 mL 0.2 M NaH<sub>2</sub>PO<sub>4</sub>, 236.75 mL 0.2 M Na<sub>2</sub>HPO<sub>4</sub>, 100 mL 3 M NaCl, 10 mL 1 M imidazole and 640 mL H<sub>2</sub>O. The elution buffer (50 mM sodium phosphate buffer pH 8.0) was prepared by mixing 6.625 mL 0.2 M NaH<sub>2</sub>PO<sub>4</sub>, 118.375 mL 0.2 M Na<sub>2</sub>HPO<sub>4</sub>, 50 mL 3 M NaCl, 125 mL 1 M imidazole and 200 mL H<sub>2</sub>O.

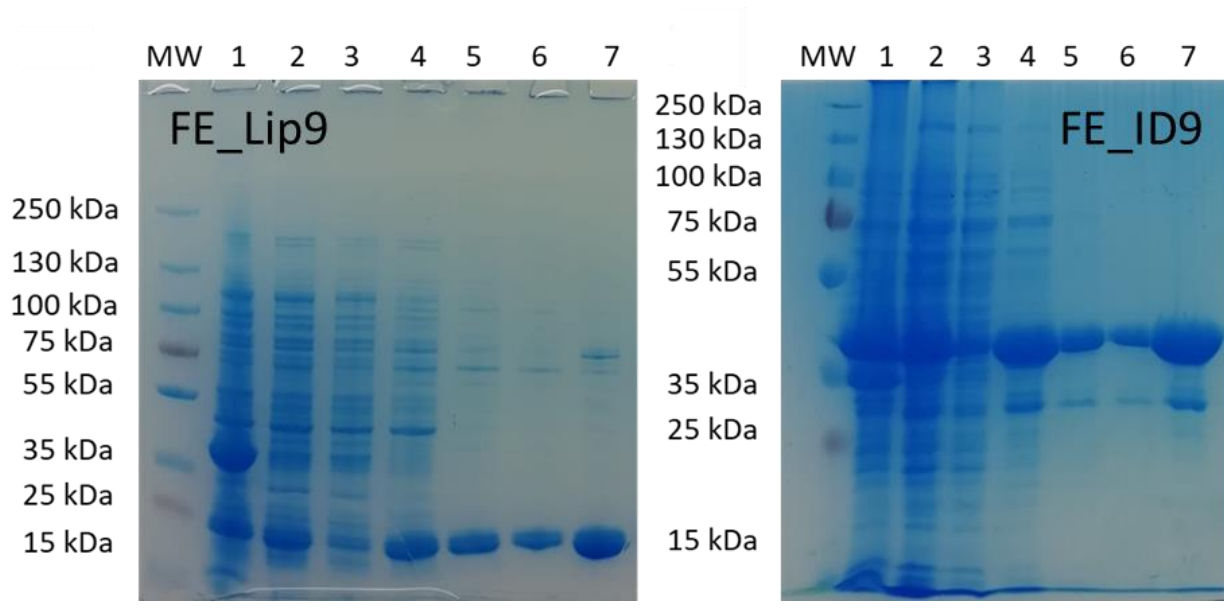
**Fermentation at milligram scale.** The FE\_Lip9, FE\_ID9 His<sub>6</sub>-tag enzymes are available in pET-45b(+) and the host *E. coli* BL21(DE3), and Kest3 and GEN0105 in p15TVL and the host *E. coli* De3 Lobstr.

- Pick one colony to inoculate 50 mL of Luria Bertani (LB) broth plus antibiotic (ampicillin [Amp] 50 µg/mL) in a 0.1-0.25-L flask.
- Incubate the culture at 37°C and 150 rpm until an OD between 0.8 - 1 (OD is measured at 600 nm).
- The next day, add this pre-inoculum to a 2.5 L-flask with 1 liter of LB + Amp 50 µg/mL and let it grow at 37°C and 150 rpm until an OD between 0.8 - 1 (OD is measured at 600 nm).
- Induce expression of the culture.
  - The plasmids pET45b(+) and p15TVL are used, which require 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) for induction.
- Incubate the culture for induction overnight at 16°C and 200 rpm.
- Centrifuge the culture at 8000 g for 30 minutes at 4°C. *Note:* the centrifugation speed and the temperature can be adapted as the enzymes are stable at room temperature.
- Resuspend the pellet in 20 mL of 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.0 to wash the pellet, centrifuged at 8000 rpm 5 minutes and at 4°C. *Note:* the buffer can be adapted as needed (for example phosphate buffer, Tris buffer, etc.), but we recommend keeping neutral pH (7.0-7.5).
- The pellet is resuspended in 20 mL of 40 mM HEPES pH 7.0. Then, Lysonase Bioprocessing reagent is added (4 µL g<sup>-1</sup> wet cells) and incubated for 60 min on ice with rotating mixing. *Note:* instead of Lysonase Bioprocessing reagent, lysozyme can be added (1 mg/mL).
- The cell suspension was sonicated using a pin Sonicator 3000 (Misonix, New Highway Farmingdale, NY, USA) for a total time of 5 min (10 W) on ice and centrifuged at 15000 g for 15 min at 4°C.
- The supernatant was retained and directly used for protein purification.

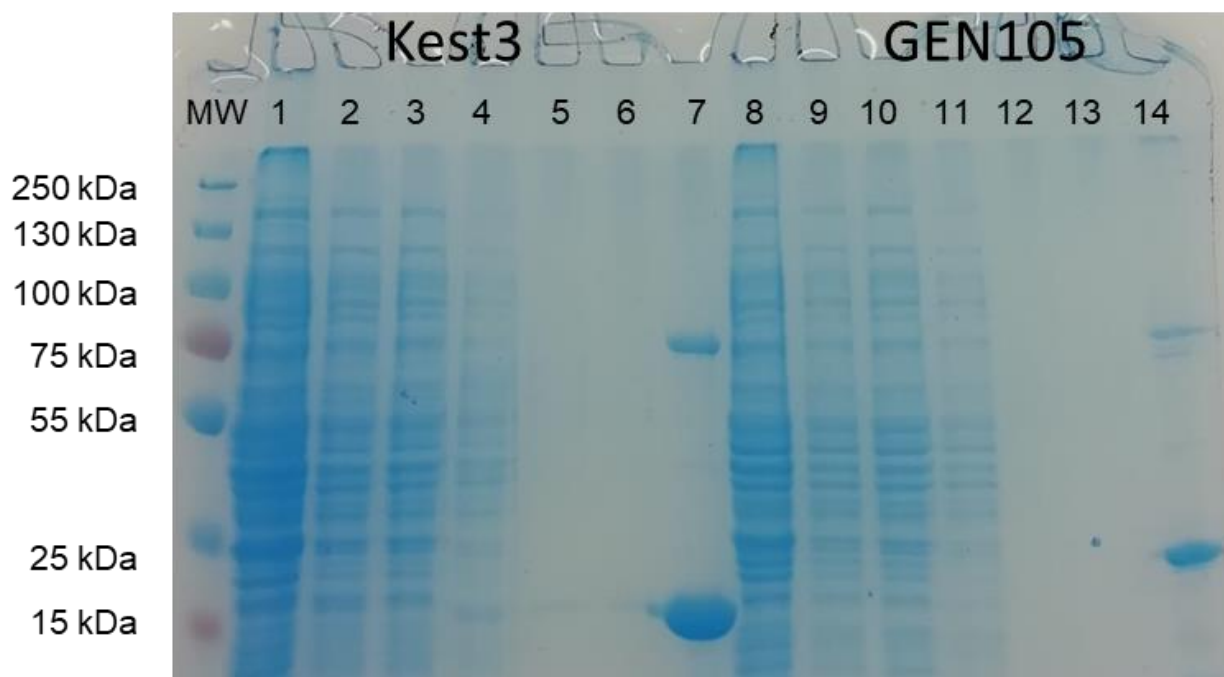
**Purification.** The His-tagged lipases were purified at room temperature after binding to a Ni-NTA His-Bind resin (Sigma Chemical Co. (St. Louis, MO, USA)). Briefly, the supernatant (50 mL) obtained following the steps detailed in the “fermentation sub-section” were mixed with an equal volume of binding buffer (50 mL). A total of 6 mL Ni-NTA His-Bind resin were added to the protein solution and the suspension maintained for one hour under gentle agitation. After incubation, the resin was separated by filtration through a 47 mm diameter glass microfibre filter (MFV1, Filter-LAB). The resin was extensively washed with washing buffer, after which the His-tagged lipases were eluted by adding elution buffer (20 mL), followed by ultrafiltration through low-adsorption hydrophilic 3000 nominal molecular weight limit cut-off membranes (regenerated cellulose, Amicon) to concentrate the protein solution. An extensive dialysis of protein solutions against 40 mM HEPES buffer (pH 7.0) and 150 mM NaCl (for FE\_Lip9) or 40 mM HEPES buffer (pH 7.0) and 200 mM NaCl (for FE\_ID9) was then performed using Pur-A-Lyzer™ Maxi 1200 dialysis kit ((Sigma Chemical Co. (St. Louis, MO, USA)), as follows. The concentrated protein solution was dialyzed against 2 L buffer during 1 h at room temperature, after which the buffer was changed by other 2 L buffer and maintained 1 h more. Then, the buffer was changed, and the dialysis was kept overnight at 4°C. The dialyzed protein solution was recovered and stored until used at -20°C.

Throughout the purification protocol, the fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels, in a Mini PROTEAN electrophoresis system (Bio-Rad), in which the proteins were stained with Coomassie brilliant blue (Protoblue Safe, National Diagnostics, GA, USA) (**Figure 1**).





**Figure 1.** SDS–PAGE analysis of the FE\_Lip9 and FE\_ID9 purification steps.



**Figure 2.** SDS–PAGE analysis of the Kest3 (lanes 1-7) and GEN0105 (lanes 8-14) purification steps.

**Degradation of stained clothes and NEFA-HR(2) kit.** Incubate the enzymes (at different concentrations) with a small piece (4 mg) of a stained cloth (purchased from Center For Testmaterials, CFT), previously cut using a circle hole puncher (5 mm diameter) in 100  $\mu$ L of HENKEL® Liquid Laundry Detergent\_A without enzymes (2.5 g/L water; see **Figure 3**), at 30°C and 950 rpm in 96-well plates. Pigment with oil on polyester/cotton PC-09 was used as target stained cloth. Two benchmark tests were performed: one with HENKEL® Liquid Laundry Detergent without enzymes (2.5 g/L water), and one with HENKEL® Liquid Laundry Detergent\_A including enzymes (3.1 g/L water; see **Figure 3**). The reaction tests are incubated and at different times (10 min, 1 h, 2 h, 4 h and 24 h) an aliquot is taken; the degradation of PC-09 was quantified by meaning of the release of non-esterified fatty acids (NEFA).



**Figure 3.** Image of the WLHUL2194600, Henkel detergent\_A Gel without enzymes, and WLHUL2161400, Henkel detergent\_A Gel including all enzymes used in this deliverable.

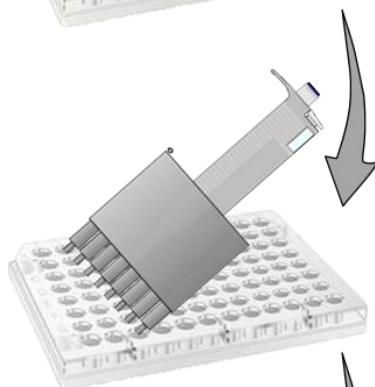
The NEFA-HR(2) kit (Waco, 517982 - Reagent 1 Set kit NEFA-HR(2) Assay, 517984 - Reagent 2 Set, kit NEFA-HR(2) Assay, 517954 - Standard, kit NEFA-HR(2) Assay), also referred to as ACS-ACOD method, is used. Briefly, Non-esterified fatty acid (NEFA) in sample is converted to Acyl-CoA, adenosine monophosphate (AMP) and pyrophosphoric acid (PPI) by the action of Acyl-CoA synthetase (ACS), under coexistence with coenzyme A (CoA) and adenosine 5-triphosphate disodium salt (ATP). Obtained Acyl-CoA is oxidized and yields 2,3-trans-enoyl-CoA and hydrogen peroxide ( $H_2O_2$ ) by the action of Acyl-CoA oxidase (ACOD). In the presence of a peroxidase, the  $H_2O_2$  formed yields a blue-purple pigment by quantitative oxidation condensation with 3-Methyl-N-ethyl-N-( $\beta$ -Hydroxyethyl)-Aniline (MEHA) and 4-aminoantipyrine (4-AA). Non-esterified fatty acids (NEFA) concentration is obtained by measuring absorbance of the blue-purple color.

The reaction outcome is tested using the NEFA-Kit, following specifications:

- Transfer 10  $\mu$ L of the reaction solution (the one detailed above) + 100  $\mu$ L of NEFA solution R1 to a 96 microplate well. Incubate 6 min at 37°C.  
*R1: Prepare R1 by mixing one bottle of Color A and Solvent A. After preparing the R1, store at 2 - 10°C and use within 1 month.*
- Add 50  $\mu$ L of NEFA solution 2. Incubate 6 min at 30°C.  
*R2: Prepare R2 by mixing one bottle of Color B and Solvent B. After preparing the R2, store at 2 - 10°C and use within 1 month.*
- Measure sample's absorbance at 550 nm using a microplate spectrometer (e.g. Synergy HT Multi Mode Microplate Reader, Agilent, Madrid, Spain).

**Figure 4** summarizes the protocol for the determination of non-esterified fatty acids (NEFA) allowing to quantify in high throughput manner the release of fatty acids from standardized stained swatches, herein exemplified by Pigment with oil on polyester/cotton PC-09 (purchased from Center For Testmaterials, CFT).

Pigment with oil on polyester/cotton PC-09



### Multi-enzyme tests

Multi-enzyme tests: Incubation with ca. 0.1 cm<sup>2</sup> PC-09 in 100  $\mu$ l HENKEL® Liquid Laundry Detergent\_A without enzymes (2.5 g/L water) plus FE\_Lip9, and/or FE\_ID9, and/or Gen0105, and/or Kest3 (0,075% w/v, each), 30°C, 950 rpm

Control test: Incubation with ca. 0.1 cm<sup>2</sup> PC-09 in 100  $\mu$ l HENKEL® Liquid Laundry Detergent\_A withenzymes (3,1 g/L in water), 30°C, 950 rpm

Transfer of reaction mix to new plate for NEFA assay



### NEFA –Kit of Wako

1. 10  $\mu$ l “wash test” + 100  $\mu$ l of R1a  $\rightarrow$  6 min, 37 °C
2. Add 50  $\mu$ l of R2a  $\rightarrow$  6 min, 30 °C
3. Measure absorbance at 550 nm

**Figure 4. Schematic representation of the microtiter plate-based assay for selecting lipases.** This method is the benchmark method to select lipases most relevant for detergent and textile sectors.

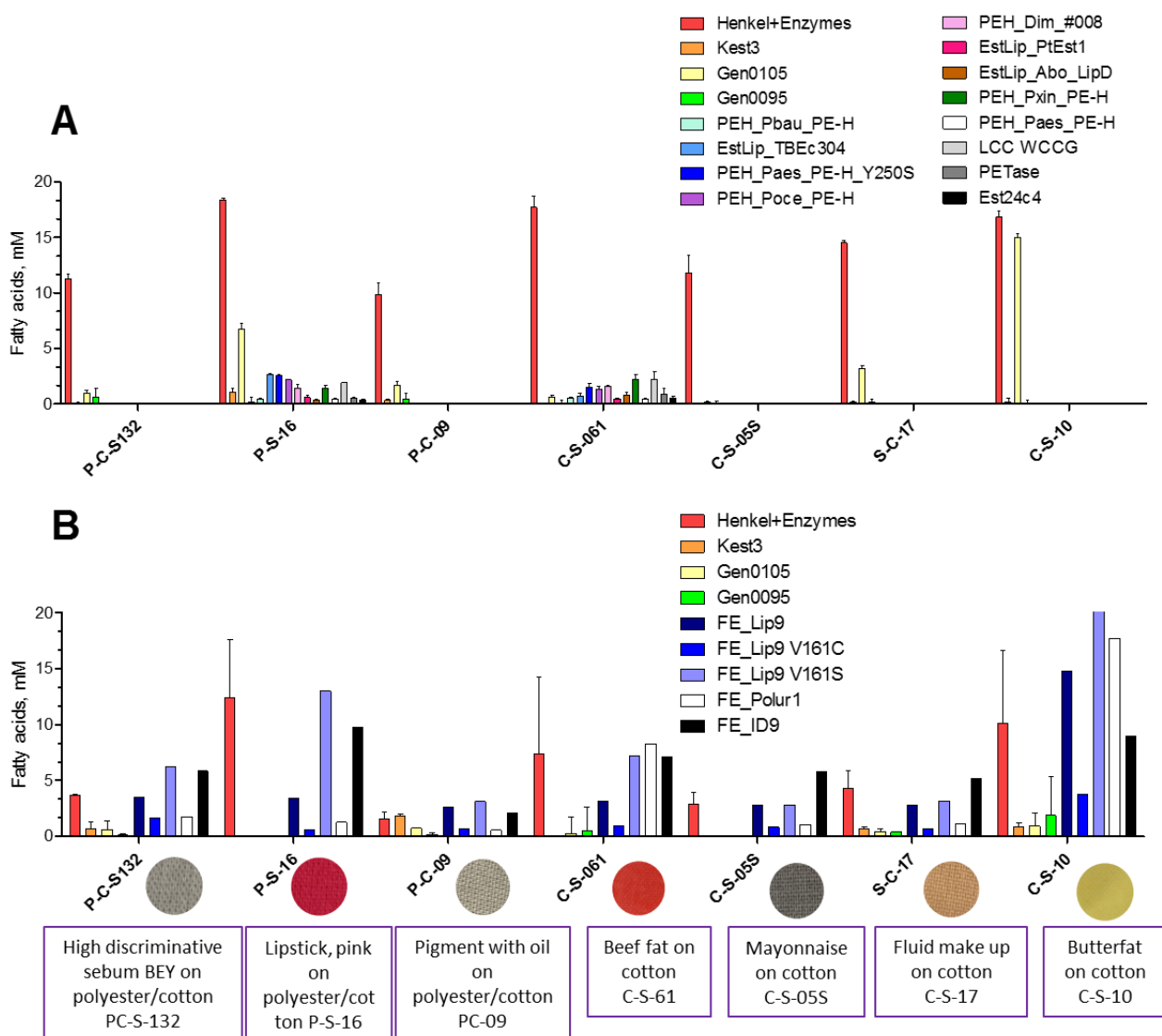
## 5. Results for multi-enzyme tests

The enzymes FE\_Lip9, FE\_ID9, Kest3 and GEN0105 were produced and purified as detailed in Section 5. After production and purification, they were added, in different combinations, to the HENKEL® Liquid Laundry Detergent without enzymes (see **D2.1**) at a concentration as low as 0.075% w/v each. Cleaning tests were performed using pigment with oil on polyester/cotton PC-09\_A and washing liquid during 10 min, 1 h, 2 h, 4 h and 24 h incubation at and 30°C; this stained fabric was used as target as it is the less degraded by the commercial HENKEL® Liquid Laundry Detergent with enzymes (**Figure 5**), which is used as benchmark (see **D2.1**).

Different combinations of the 4 selected lipases were used when added to HENKEL® Liquid Laundry Detergent without enzymes. As shown in **Figure 6**, all mixes showed higher degradation (average 2.0-fold) of PC-09 compared to the HENKEL® Liquid Laundry Detergent with enzymes, with the best combination being FE\_Lip9+Gen0105 (2.4-fold) at short incubation times and FE\_Lip9+Kest3 at long incubation times (4-24 h). In addition, when comparing the data obtained for single enzymes (**Figure 5A**) and multi-enzyme cocktails (**Figure 6**), we observed that the multi-enzyme cocktails degrade of PC-09 at higher levels compared to the single enzymes.

The results presented in this deliverable demonstrate that the first combinations tested provided higher washing efficiencies compared to single enzymes and compared to benchmark HENKEL® Liquid Laundry Detergent\_A including enzymes. Based on the initial results in the experimental validation for the pre-selected enzymes, we are now running a second round of multi-enzyme tests to refine the search of optimal combinations. For this second round we will include all priority enzymes detailed in Section 3, and improved

variants generated by protein engineering (Tasks 5.1-5.3) and by supramolecular engineering through immobilization and organosilica shielding (Task 5.4).

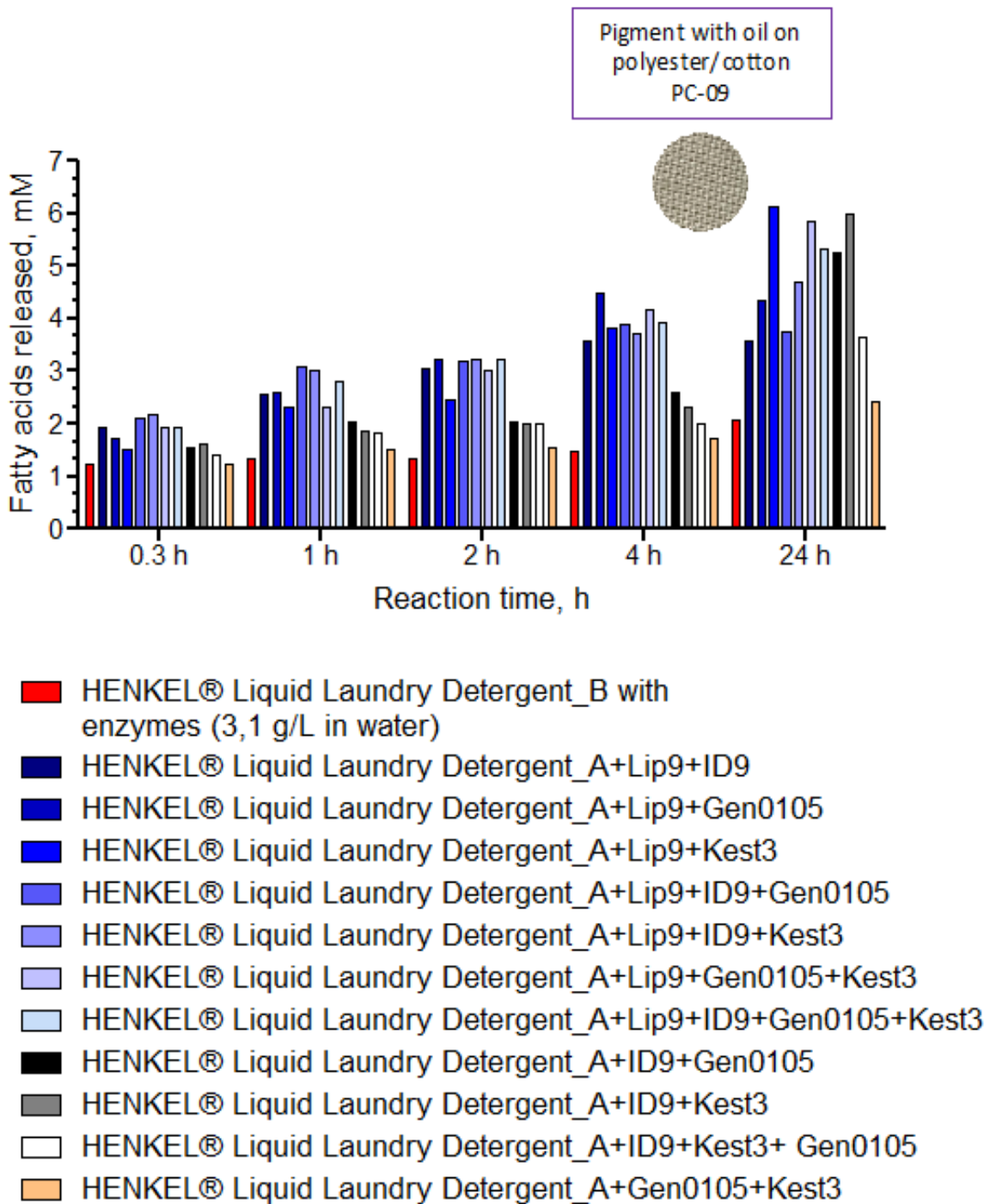


**Figure 5. Enzymatic preparations capable of degrading stained swatches.** Tests were performed as detailed in D3.2 using a buffer (A) or washing buffer (B). As a reference the commercial HENKEL® Liquid Laundry Detergent with enzymes

For this second round all priority stained swatches, synthetic fabrics and dye detailed in D2.1, will be used.

- C-S-61, Beef fat, colored with Sudan red on Cotton - 45 cm width
- PC-09, Pigment with oil on Polyester/Cotton - 90 cm width
- C-S-05S, Mayonnaise with carbon black on Cotton - 90 cm width
- C-S-10, Butterfat with colorant 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 on Polyester/Cotton - 45 cm width
- Synthetic materials 61488, 61488Z ROH, 61488Z VORB, 92% PA, 8% EL 180g/m<sup>2</sup>
- Synthetic materials 61988, 61988F1 ROH, 61988F1 VORB, 92% PA, 8% EL 280g/m<sup>2</sup>
- Synthetic materials 67007, 67007 ROH, 67007 VORB, 88% PA, 12% EL 135g/m<sup>2</sup>
- Synthetic materials 3X58, 2X34G ROH, 3X58 VORB, 100% PES 100g/m<sup>2</sup>

- Synthetic materials 66299, 5237/00 ROH, 92% CO, 8% EL 240g/m<sup>2</sup>
- Synthetic materials E03130, E03130 ROH, E03130 VORB, 80%PA6, 20%EL
- Real-life dye BEMAPLEX Black D-HF



**Figure 6. Degradation of stained swatch by multi-enzyme cocktail.** Pigment with oil on polyester/cotton PC-09\_A was used as a reference stained swatch. Different combinations of enzymes were used, all added at a concentration of 0.075% each to the commercial HENKEL® Liquid Laundry Detergent without enzymes. As a reference the commercial HENKEL® Liquid Laundry Detergent with enzymes. Tests were performed as detailed in D3.2 using washing buffer. At different incubations times samples were taken and the release of fatty acid was quantified as detailed in D3.2.