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REPORT ON FERMENTATION, DSP AND ACTIVITY VERIFICATION FOR 18 PRELEAD ENZYMES

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1. Scope of Deliverable

This report provides a summary of the development of fermentation methods and the characterization of pre-lead enzymes produced on a multi-gram scale. The pre-lead enzyme candidates, which include recombinant, native, and biomimetic variants, have been produced in quantities sufficient for validation tests that precede pre-industrial trials. These enzymes have been produced and subsequently shared with partners for pre-industrial validation testing. The report details the production processes of these lead candidates, focusing on their synthesis in native hosts, *Escherichia coli*, and *Pichia pastoris*.

2. Introduction

As mentioned in deliverable D6.1, deliverable D6.2 is also a continuation of Deliverable D5.1, 'The Shortlist of at Least 18 Enzymes Nominated for Engineering,' in which a set of enzymes were selected based on laboratory experimental results for their potential scalability and testing in three relevant applications to the project: detergents, textiles, and cosmetics. In this phase, two production methods were chosen: one using Escherichia coli and the other using Pichia pastoris. For the production phase a total of 41 enzymes have been selected for scale-up production until now (M32), as detailed in Deliverable D6.1. Following the production phase, we proceeded with experimental validation to assess the levels of production and to evaluate the activity and/or stability of the enzymes. This step was crucial before moving forward to their use by our industrial partners.

3. Production of enzymes in *Escherichia coli* (12 L scale)

The focus was on two lipases, FE_Lip9 and FE_ID9, identified as prime candidates for large-scale production. The initial production attempts for these enzymes utilized Pichia pastoris expression systems, but challenges arose. For FE_Lip9, production in Pichia pastoris, undertaken by BioSynth, yielded low expression levels in the initial batches. This led to a shift to an *Escherichia coli* expression system, which successfully facilitated gram-scale production of the enzyme. Similarly, initial attempts to express FE_ID9 in *Pichia pastoris* were not successful. Consequently, an Escherichia coli system was employed, which proved effective for the large-scale production of this enzyme.

3.1. Materials for production in Escherichia coli (12 L scale)

Materials. His_6-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). 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 imidazol) was prepared by mixing 13.25 mL 0.2 M NaH_2PO_4, 236.75 mL 0.2 M Na_2HPO_4, 100 mL 3 M NaCl, 10 mL 1 M imidazol and 640 mL H_20. The elution buffer (50 mM sodium phosphate buffer pH 8.0) was prepared by mixing 6.625 mL 0.2 M NaH_2PO_4, 118.375 mL 0.2 M Na_2HPO_4, 50 mL 3 M NaCl, 125 mL 1 M imidazol and 200 mL H_2O.

Equipments. Sonicator 3000 (Misonix, New Highway Farmingdale, NY, USA). Fermenter Biostat C Plus (B. Braun Biotech): Automatic Stirred Reactor (STR); working volume 30 litres; PDI online control of temperature, pH, agitation and foaming; air mass flow control; hierarchical dissolved oxygen control; sterilisable in situ; software: MFCS/win3.0 (**Figure 1**). French Press (GEA Niro Soavi): Working pressure, 2000 bar; Working flow rate, 150 mL/minute.

3.2. Materials for production in Escherichia coli (12 L scale)

Fermentation at gram scale. The FE_Lip9 and FE_ID9 His_6-tag enzymes are available in pET-45b(+) and the host *E. coli* BL21(DE3): (i.) One colony is picked and used to inoculate 80 mL of Luria Bertani (LB)[1] broth plus antibiotic (ampicillin [Amp] 50 µg/mL); (ii.) The cultures were then incubated at 37°C overnight; the final OD is about 2.2 (OD is measured at 600 nm); (iii.) The next day, add this pre-inoculum to a 12 L-Biostat C Plus fermentor (**Figure 1**) with auto-induced broth medium + Amp 50 µg/mL and let it grow at 37°C until an OD of 0.9 (OD is measured at 600 nm); (iv.) Leave the culture overnight; (v.) Centrifuge the cultures; (vi.) The pellet (about 69 grams) is resuspended in 286 mL of 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.0; (vii.) Cells are lysed by French Press (**Figure 1**), after which the suspension was centrifuged at 11000 rpm, 20 min, 4°C; (viii.) The supernatant was retained and directly used for protein purification.

Figure 3. Fermenter Biostat C Plus (B. Braun Biotech) and French Press (GEA Niro Soavi) used for the production of lipases FE_Lip9 and FE_ID9 batches at gram scale.

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 Hig-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 Comassie brilliant blue (Protoblue Safe, National Diagnostics, GA, USA).

Activity tests**.** Hydrolytic activity was determined using appropriated spectrophotometers and analytical techniques detailed in deliverable D3.2 "Standard assays, analytics and calculations for monitoring enzymatic performance".

3.3. Results for production in Escherichia coli (12 L scale)

The production of FE_Lip9 and FE_ID9 His_6-tag lipases was undertaken at 12-L scale using an *E. coli* expression system that allow production of intracellular enzyme. Previously, the expression was tested in Erlenmeyer

flasks at 1-L scale. As shown in **Figure 2**, both lipases were produced in soluble form when produced in 1-L fermentation scale and semi-purification by binding to a Ni-NTA His-Bind resin. A total of 10.2 mg and 63.3 mg of FE_Lip9 and FE_ID9 His_6-tag lipases, respectively, were obtained from 1-L culture.

As detailed in D5.1 "The shortlist of at least 18 enzymes nominated for engineering", the pure lipase preparations prepared at milligram scale did show activities of interests and for this reason their production at gram scale was undertaken. Following the protocol detailed in section 3.2, the production was scaled up to 12 L (**Table 2**), being able to obtain a total of approx. 3.96 and 10.2 g of FE_Lip9 and FE_ID9 His_6-tag lipases, respectively, from the 10 L cultures. A purity higher than 95% was achieved as shown in **Figure 3**.

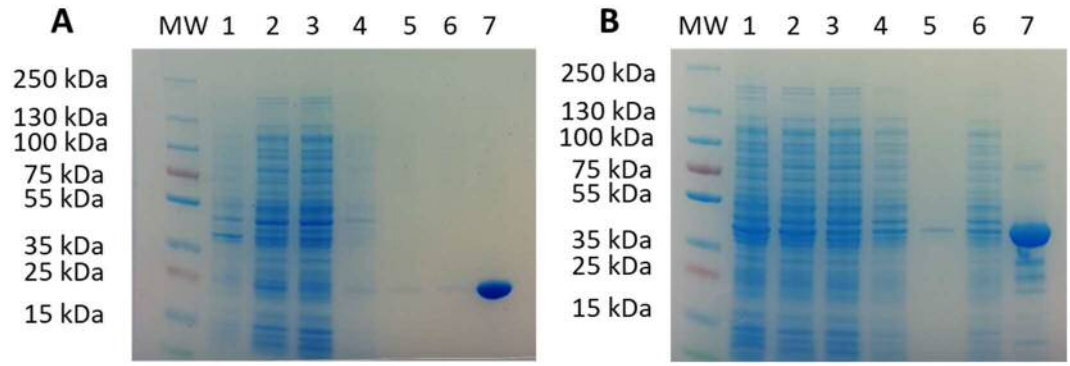


Figure 2. SDS–PAGE analysis of the FE_Lip9 (panel A) and FE_ID9 (panel B) His_6-tag lipases, prepared at 1L-fermentation scale, using a 12% Tris-glycine SDS-polyacrylamide gel. Panel A: MW, molecular weight marker; lane 1, unsoluble protein extract; lane 2, soluble protein extract; lane 3, soluble protein not bind to Ni-NTA His-Bind resin; lane 4-6, proteins eluting from the Ni-NTA His-Bind resin during the washing step with washing buffer; lane 7, pure protein. Panel B: MW, molecular weight marker; lane 1, unsoluble protein extract; lanes 2-3, soluble protein extract; lane 4, soluble protein not bind to Ni-NTA His-Bind resin; lane 5-6, proteins eluting from the Ni-NTA His-Bind resin during the washing step with washing buffer; lane 7, semi-pure protein.

Table 2. Fermentation and purification yields for FE_Lip9 and FE_ID9 His_6-tag lipases.

Name	FE_Lip9	FE_ID9
Total cell pellet, grams	10.2 g	24.7 g
Volume, after French Press	200	180
Total protein (mg/mL), after French Press	23.31 mg/mL	63.26 mg/mL
Total protein, after His-tag purification	1051 mg	2009 mg

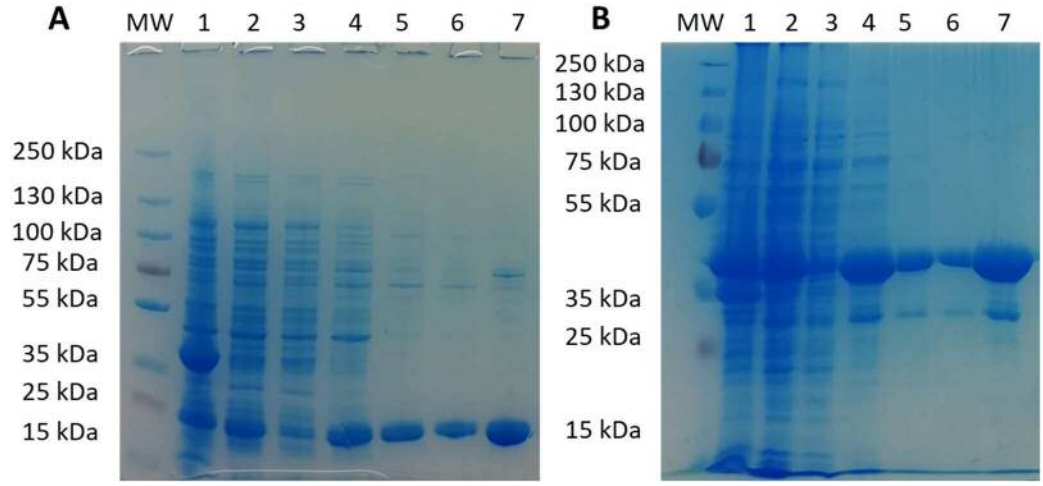


Figure 3. SDS–PAGE analysis of the FE_Lip9 (panel A) and FE_ID9 (panel B) His6-tag lipases, prepared at 12-L fermentation scale, using a 12% Tris-glycine SDS-polyacrylamide gel. Panel A: MW, molecular weight marker; lane 1, proteins extracted from cell pellet after French Press; lane 2, soluble protein extract; lane 3, soluble protein not bind to Ni-NTA His-Bind resin; lane 4-6, proteins eluting from the Ni-NTA His-Bind resin during the washing step with different concentrations of elution buffer; lane 7, pure protein. Panel B: MW, molecular weight marker; lane 1, proteins extracted from cell pellet after French Press; lane 2, soluble protein extract; lane 3, soluble protein not bind to Ni-NTA His-Bind resin; lane 4-6, proteins eluting from the Ni-NTA His-Bind resin during the washing step with different concentrations of elution buffer; lane 7, pure protein.

The FE_Lip9 and FE_ID9 His_6-tag lipases purified at gram scale were transferred to partners on 23 November 2022 for their supramolecular engineering and testing.

4. Production of enzymes in native host: *Vibrio alginolyticus* IAMC-CNR#23

The strain *Vibrio alginolyticus* IAMC-CNR#23 was received by IAMC-CNR of Messina. An initial screening of growth media from *BCSMed Dat* was performed with the strain. The bacterium was grown overnight in Marine Broth (MB) agar plates and then used the obtained sufficient biomass to inoculate different media (100 mL in 500 mL flasks). Growth was carried out at 22°C and 200 rpm. From this initial screening fermentation media BCS365 and BCS366 were identified as suitable for the growth of *Vibrio alginolyticus*. The selected media were used for initial trials of hyaluronidase production. Liquid cultures of *Vibrio* #23 in BCS365, BCS366 and Marine Broth (as control) were prepared as previously described. Fermentations were run up to 72 hours. Bacterial growth (turbidimetry) and hyaluronidase activity were monitored every 24 hours. Hyaluronidase activity was assayed by measuring the reduction of the turbidity resulting from complexes formed between hyaluronic acid and BSA. The enzymatic activity (secreted activity) was searched on cultures supernatants obtained after centrifugation of cultures samples at 16000g for 10 minutes. Reduction in turbidity was obtained for all three samples with supernatants from BCS366 cultures being the best (**Figure 4**).

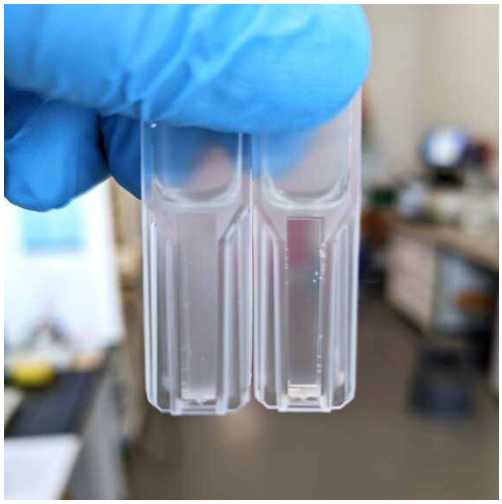


Figure 4. Turbidity assay, blank cuvette with sample treated with water and cuvette with sample treated with BCS366 culture supernatant.

BCS366 grown cultures also gave the best results regarding growth with an OD_{600} of 8.6. For this reason, BCS366 was selected for the optimization of hyaluronidase production (Figure 5).

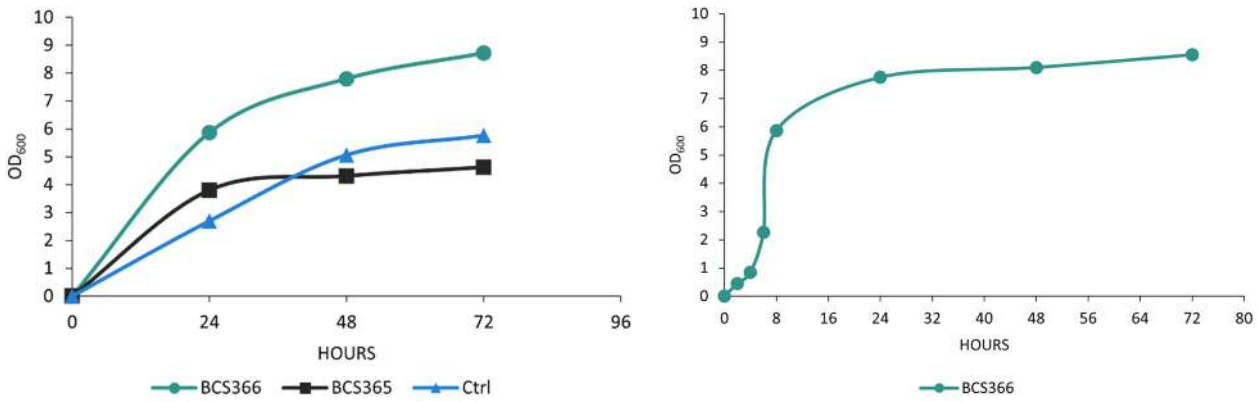


Figure 5. Vibrio #23 OD_{600} in BCS336, BCS365 and MM (left), and detail of the growth kinetic in BCS366 (right). Data reported are the average of 2 independent experiment.

4.1. Scale-Up to 15 liters and downstream production of hyaluronidase from Vibrio alginolyticus IAMC-CNR#23

A first 15L fermentation was carried out using BCS366 as the production medium. The medium was prepared without hyaluronic acid as inducer. To inoculate the bioreactor four shake flask cultures were prepared using cell loops grown overnight at 25°C on MB agar plates. After 24 hours of incubation at 22°C and 200 rpm, three flasks were pooled together and used as the inoculum.

The fermentation was carried at 22°C and 400 rpm. In these conditions the strain reached an OD_{600} of 10.5 after 20 hours of incubation. The obtained fermentation broth was centrifuged for 60 minutes at 7200g. The supernatants were then pooled together, decanted, and finally filtered through filter paper.

Ultrafiltration on 30 KDa cellulose membrane was performed to concentrate extracellular proteins. The solution was concentrated 17x. The obtained sample was loaded on SDS-PAGE gels (3.5% acrylamide stacking and 11% resolving) to verify the presence of proteins with molecular weight ranging from 80 to 100 kDa. Coomassie staining revealed the apparent presence of two proteins of 30-37 kDa without any visible bands in the hyaluronidase molecular weight range (**Figure 6**) (despite hyaluronidase activity was observed). It is very likely that hyaluronic acid may be needed in the fermentation broth to better induce the expression of enzymes with hyaluronidase activity. Considering the possibility of having inducible enzymes, shake flask fermentations in the presence of different inducers were performed. In particular, hyacare 100 and 50, cellobiose and chitin were tested for the induction of hyaluronidases in *V. alginolyticus* liquid cultures. Hyacare 100 and 50 were added to the culture medium in the concentration of 0,25 g/L while cellobiose and chitins were tested in the concentrations of 0,25 and 2,5 g/L. Liquid cultures were prepared as described above. The cultures were all incubated at 22°C and 200 rpm besides the cultures with hyacare 100 which were also incubated at 37°C.

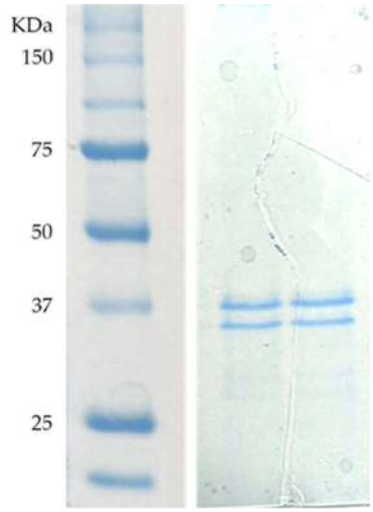


Figure 6. SDS-PAGE analysis of concentrated culture supernatants of Vibrio #23

Fermentations were carried out for 48 hours. The harvested cultures were centrifugated and supernatants concentrated on 30 kDa Microcon centrifugal filter unit. The 10x concentrated samples were then loaded on SDS-PAGE gels to verify the presence of proteins with molecular weight in the desired range. As evidenced in **Figure 7**, Coomassie staining developed smear for samples 5, 6 and 7 indicating the possibility of having proteins in the desired molecular weight range. Therefore, Hyacare 100 may be the best inducer for hyaluronidase production in *Vibrio #23.* For this reason, pilot-scale production was tested using hyacare 100 as hyalyuronidase inducer.

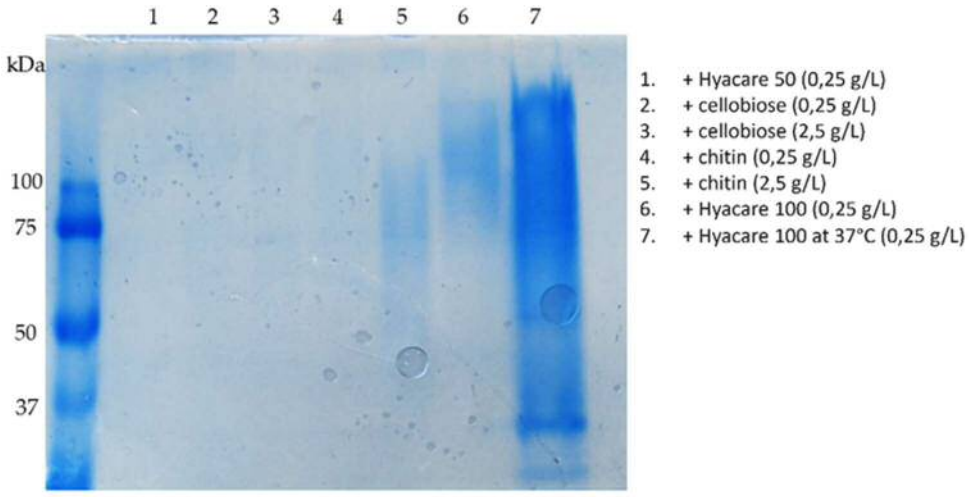


Figure 7. SDS-PAGE analysis of concentrated culture supernatants of Vibrio #23 in the presence of different possible inducers.

4.2. Production of hyaluronidase at the 15L scale with hyacare 100 induction

Large scale production was performed using BCS366 and hyacare 100 0,25 g/L as hyaluronidase inducer. For this purpose, 15L of production medium were prepared and sterilized at 121°C for 20 minutes. The inoculum was prepared from 5 100-ml cultures of *Vibrio #23* grown on BCS366 medium for 24 hours at 22°C and 200 rpm. The cultures were pooled together and used to inoculate 15L of BCS366. A solution of 37,5 g/L of hyacare 100 was finally added to the bioreactor to achieve a final concentration of 0,25 g/L. The fermentation was carried out at 37°C and 400 rpm. Bacterial growth was monitored after 20 hours of incubation by means of turbidimetry. In this condition, the OD_{600} reached resulted of 7.5. To avoid possible cell lysis the 15L culture was therefore harvested.

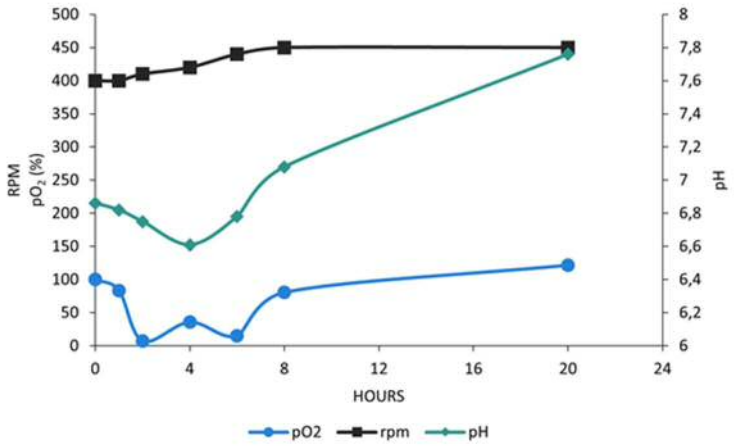


Figure 7. fermentation trends of rpm, pH and pO_2.

The separation of the bacterial cells from the supernatant was performed at 7200g for 60 min. The hyaluronate lyase-containing supernatant was then decanted and filtered through filter paper prior to ultrafiltration. Concentration of the supernatant containing the hyaluronate lyase activity was performed through ultrafiltration on 30 kDa cellulose membrane. The concentration achieved was 23x. The resulting concentrate appeared to be very viscous probably due to the presence of residual hyaluronic acid. It was interesting to note that the viscosity did not decrease over time suggesting partial dilution of HA and the

presence of stable degradation products. SDS-PAGE analysis was performed on denaturing gels 3.5% acrylamide stacking and 12% resolving. As shown in **Figure 8,** in the size range expected for hyaluronate lyase, a protein smear appeared. This could be due to the presence of residual hyaluronic acid or to the glycosylation of the hyaluronate lyase(s). High salinity of the sample was excluded as the same result was obtained upon precipitation of the protein with 70% ammonium sulphate. The treatment of the secretome with glycosidases is in progress to elucidate this issue. To reduce its viscosity, the solution was gradually acidified with HCl 6N to hopefully precipitate the residual hyaluronic acid. From pH 4 to pH 1 precipitates were gradually obtained. The obtained samples were then centrifuged, and supernatants loaded on SDS-PAGE gels. Protein smears disappeared from samples collected at acidic pH (4 to 1) while remained present in samples at pH 5, 6, 7 and 8.

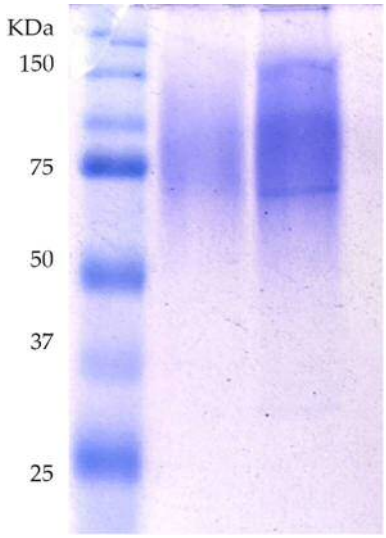


Figure 8. SDS-PAGE analysis of culture supernatant (1), protein concentrate 23x (2).

4.3. Conclusions and outlook for the scale-up to 15 liters and downstream production of hyaluronidase from Vibrio alginolyticus IAMC-CNR#23

The production of hyaluronidase from V. alginolyticus was carried out in medium BCS366. The overall yield of secreted proteins, with hyaluronidase being the most abundant, was approximately 0.1 g/L, resulting in a total productivity of 1.4 grams. The detected hyaluronidase activity was sufficient for the degradation of hyaluronic acid, facilitating the verification of the resulting hyaluronic acid digestion products. Efforts to improve productivity and the assay method are currently underway. Samples produced as described were delivered to selected partners. The samples were provided in both liquid and lyophilized forms. It is interesting to note that the activity was retained in both the liquid samples stored at 4°C and the lyophilized samples.

5. Production of enzymes in *Pichia pastoris*

The yeast *Pichia pastoris* wild type was used for the expression of the identified lead candidates. The main steps are summarized below:

- Sequence optimization, synthesis and cloning of the different target genes, testing different signal peptides.
- Transfection in the *Pichia pastoris* wild type, and clones screening in 24 well-plate.
- Identification of the best candidates and fermentation in 10 L scale.
- Purification with ultrafiltration and diafiltration.

- Final confirmation of the activity using reference substrates

In the following, the process of optimizing the gene sequences, generation of stable integration clones, screening for the best-expressing clone and production of the pre-lead candidate in 10 L scale are described in general, and examples of the results are shown for one of the candidates.

5.1. Gene design, synthesis and cloning of the different pre-lead candidate genes

The gene sequences, originally provided by the consortium partners in work package 4, were optimized for expression in the methylotrophic yeast *Pichia pastoris* and cloned into the Biosynth-owned plasmid "pPichia57", the DNA synthesis was performed by an external service provider. This plasmid is designed for the stable genomic integration, into the *Pichia pastoris* AOX locus, of the expression cassette containing the target gene and a Zeocin (Zeo) resistance marker. The antibiotic selection (Zeo) is only utilized for the initial clone selection, while not required in the culture media during the later protein expression. Due to the stable genomic integration, the recombinant expression is induced and maintained by addition/feeding of methanol.

For transformation of *Pichia pastoris* and selection of stable clones, Biosynth standard protocols were used. After overnight digestion of 7.5-10 µg of plasmid DNA with restriction enzyme PmeI to linearize the DNA for higher integration efficacy, electro-competent *Pichia* cells were prepared from a fresh overnight culture. The cells were finally resuspended in 1M ice-cold sorbitol and immediately used for electroporation. Following a 2h regeneration phase, the transformed cells were plated of dilutions of 1:500, 1:5 000 and 1:50 000 on a series of YPD plates containing 150/900/1400 mg/L of the antibiotic Zeocin for selection of transformants. When colonies became visible after approx. 3 days, such clones were re-streaked to confirm the ability to grow on the corresponding Zeocin concentration.

5.2. Small scale-screening of integration clones

The screening of the yeast transfectants was performed in sterile 24 well-plates, closed with a sterile air-permeable septum-lid. 2.5 mL BMxY media was added to each well of the 24-well plates and 11 transfectants pro construct were singularly inoculated in each single well. A wild-type colony was also inoculated as negative control. 25 µL MeOH (100 %) was added to each well of the plate. The 2.5 ml 24-well expression cultures were incubated at 28°C at 250 rpm for 4 days.

30 µl MeOH (100%) was added to each well, once in the morning and once evening (~9-12 hours apart) during the following next 3 days, for a total culture time of ~96 h. The expression culture supernatants were then harvested by centrifugation (20 min, 10000 rcf, 4°C).

5.3. Small scale-screening: activity test and enzyme detection

To identify the best candidate for the large-scale expression in 10 L fermenter, aliquots of the collected supernatant were used for the determination of the enzyme activity and of the protein content. The target enzyme detection was done with Coomassie staining.

Enzyme activity (U/mL) was determined against the substrate pNP-butyrate in 50 mM Tri-HCl pH 7.5. **Figure 9** shows the analysis for the enzyme PEH_Poce_PE-H.

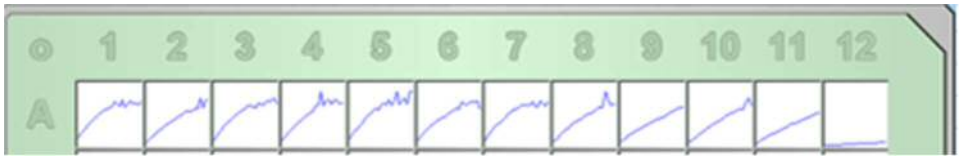


Figure 9 – Exemplarily activity kinetic of the enzyme PEH_Poce_PE-H. Kinetic measurements were conducted by measuring absorbance at 405 nm every 42 seconds for 17 min at 37 °C, in 96 Microtitre plate.

The most active supernatants were then concentrated with 3 kDa centrifugal filters and loaded on 4-20% TGX-SDS-PAGE gel. 3 µL of a 5x Laemmli SDS-PAGE loading buffer were added to 12 µL of the concentrated supernatants. The samples were then incubated at 95 °C for 5 min and loaded on a SDS-PAGE gel (see example for PEH_Poce_PE-H in **Figure 10**).

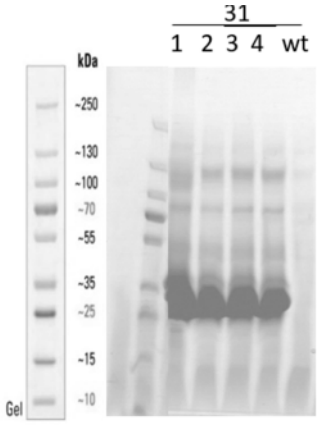


Figure 10 – Exemplarily SDS-PAGE for the enzyme PEH_Poce_PE-H.

The best candidates of each pre-lead enzyme candidate were then selected for expression in 10 L scale fermentations using a Biosynth standard protocol for high cell density fermentations.

5.4. Production of pre-lead candidates in 10 L scale

Pichia pastoris strains were cultivated in Basalt Salts Media (BSM) containing glycerol as sole carbon source. The induction phase for the recombinant protein expression was performed by a pulsed methanol feed of 20-25 pulses. Induction temperature was fixed at 25°C for all *Pichia pastoris* fermentations for the different pre-lead enzymes (see example for PEH_Poce_PE-H in **Figure 11**).

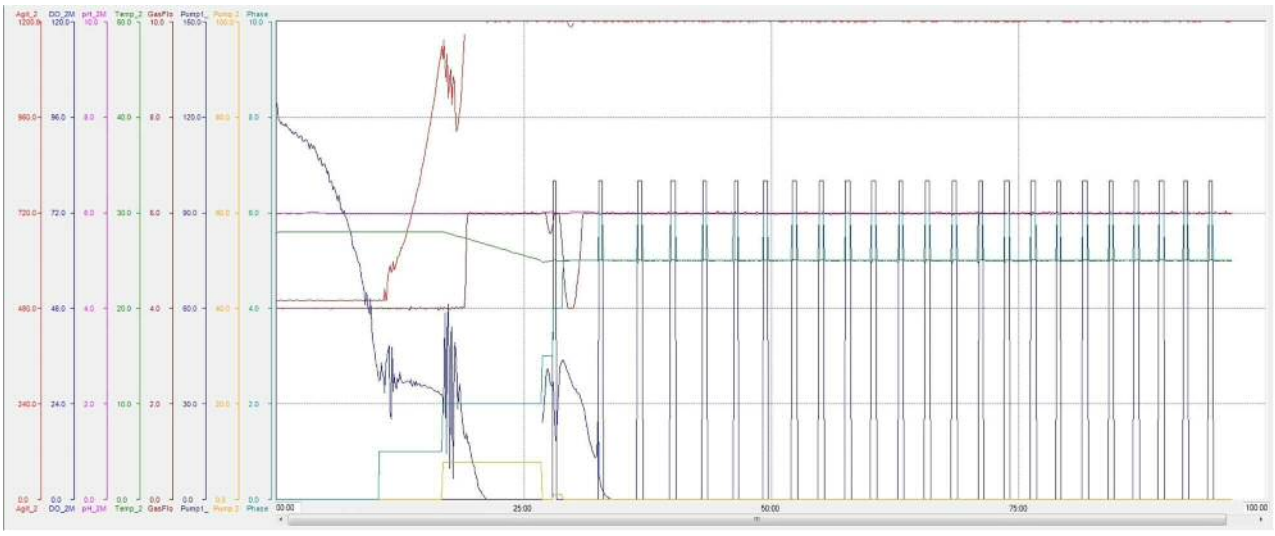


Figure 11 – Exemplarily fermentation diagram for the expression of the enzyme PEH_Poce_PE-H. Control parameters and cultivation phases are indicated on the left side in different colors and recorded for all cultivation length.

All *Pichia pastoris* culture supernatants were harvest after fermentation end and further processed for buffer exchange and lyophilization of the resulting pre-lead enzyme solutions. After harvesting by centrifugation, the fermentation supernatant was filtered with 0.2 µm hollow fiber filter module for the removal of residual cell debris and the permeate buffer exchanged into 25 mM KPi, 50 mM NaCl, pH 6.3 formulation buffer. The

retentate was lyophilized at -20°C, 20 µbar, for 72 hours. The powder is then collected and stored at 4°C, and a small aliquot used for specific activity, protein content and size (SDS-PAGE) verification.

5.5. Confirmation of the expression and activity

21 mg/mL of the powdered enzyme were resuspended in water. The enzyme solution was then used for the final enzyme activity measurement with pNP-butyrate, protein content with the Bradford quantitation assay, and Coomassie staining for enzyme detection.

Samples from the different purification step were collected and loaded onto a 4-20% SDS-PAGE (see example for PEH_Poce_PE-H in **Figure 12**).

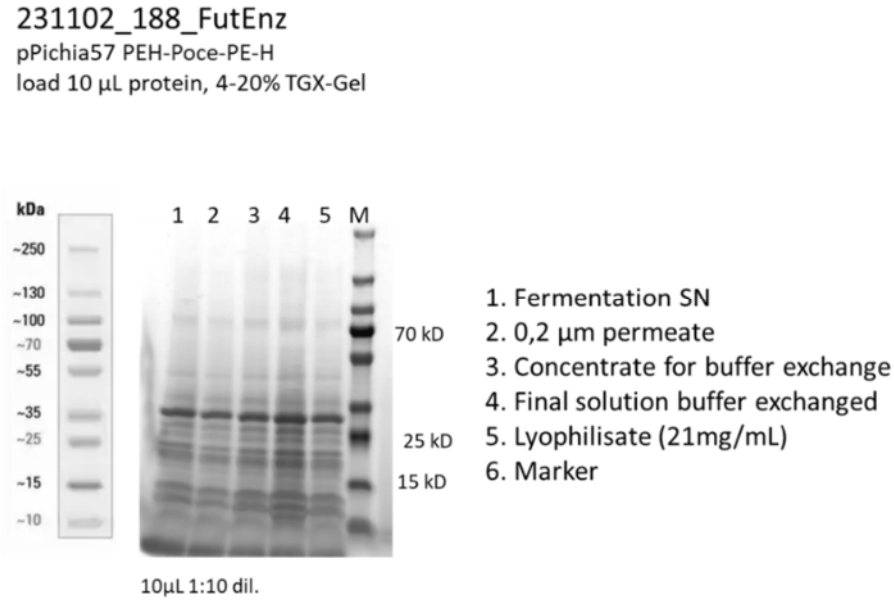


Figure 12 – Exemplarily SDS-PAGE of the PEH_Poce_PE-H purification steps' samples and final lyophilized product. Fermentation supernatant, microfiltration, ultrafiltration, buffer exchanged and resuspended powdered enzyme samples were loaded onto an SDS-PAGE.

Protein content determination

The total protein content was determined with the Bradford quantitation assay, by measuring absorbance at 595 nm. Liquid protein samples are diluted in 1:3 serial dilutions and the calibration standard is diluted from commercial BSA (see example for PEH_Poce_PE-H in **Figure 13**).

	AVG [mg/mL]	STD
supernatant	3,61	0,8
microfiltration	2,38	13,79
ultrafiltration	4,37	3,07
lyophilized sample	4,49	1,3

Figure 13 – Exemplarily protein content determination of the PEH_Poce_PE-H purification steps' samples and final lyophilized product.

Activity assay

The activity of the enzyme candidates was confirmed with a reference substrate in a standard assay, e.g. using pNP-butyrate for the lipases/esterases/PETases. The hyaluronidases were tested using the consortium assays.

5.6. Conclusions and outlook for the scale-up production in Pichia pastoris

In work package 6, the consortium partners have successfully developed fermentation methods to produce the pre-lead candidates at larger scale.

While Biosynth (formerly Eucodis Bioscience) has mainly focused on the secreted expression of the pre-lead candidates in *Pichia pastoris*, CSIC used *E. coli* for the production of some of the respective target enzymes. BioChem Solutions on the other hand contributed with their experience in fermentation of native stains, and produced some enzyme candidate like hyaluronidases which were difficult to obtain in the other expression systems. The produced enzymes were then supplied to the project partners for confirmation of the activity and further characterization as well as for industrial application tests by the industrial partners.

6. Verification of activity of enzymatic materials produced at multi-gram scale

In the section below, titled 'Activity Verification Results,' we provide a summary of the data on activity verification. This includes tests conducted on both model substrates and pre-industrial validation substrates. The enzymes listed in the following table are organized by a number (#XX), corresponding to their sequence in the 'Enzyme Specification Sheets' included in this report. These sheets detail each enzyme's name, applications, measurement conditions, substrates used, and additional data that demonstrate their activities and properties.

7. Final remarks

In light of the comprehensive findings and detailed analyses presented in this report, it is clear that the objectives of the Deliverable D6.2 (or D33) outlined at the onset of this project have been successfully met. A copy of the submitted Deliverable D6.2 (or D33) has been recorded in the intranet's project website. See www.futurenzyme.eu -> login -> private-area -> DELIVERABLES & MILESTONES -> DELIVERABLES -> D6.2_Report on fermentation, DSP and activity verification for 18 Pre-Lead enzymes.

Activity Verification Results Brief

In this report, we summarize the data on activity verification against model substrates and/or pre-industrial validation substrates. The enzymes in the table below are ordered by a number #XX, which corresponds to their order of appearance in the "Enzyme Specification Sheets" detailed in the report. These sheets include the name of the enzyme, its applications, measurement conditions, substrates, as well as additional data demonstrating their activities and properties.

Number[1]	Enzyme	Producer	Batch	Activity*
#01	EstLip-Dim#008[1]	BIOSYNTH	03914023SC0824	Verified
#02	EstLip-Paes-TB035[1]	BIOSYNTH	03908323SS0521	Verified
#03	Pform_PE-H[1]	BIOSYNTH	03917823SS1013	Verified
#04	GEN0105[1]	BIOSYNTH	03920023SS1120	Verified
#05	pVec11[1]	BANGOR	-	Verified
#06	EstLip_TBEc304[1]	BIOSYNTH	03917723SS1006	Verified
#07	Paes_PE-H Y250S[1]	BIOSYNTH	03908423SC0609	Verified
#08	PtEst[1]	BIOSYNTH	03911523SC0728	Verified
#09	FE_Polur1[1]	BIOSYNTH	03911323SC0726	Verified
#10	FE_EH37[1]	BIOSYNTH	03919423SS1116	Verified
#11	FE_Lip9[1]	BIOSYNTH/CSIC	03908223SS0516	Verified
#12	Hyal_HRDSV_2334[1]	BIOSYNTH	03920323SS1127	Verified
#13	*Vibrio alginolyticus* #23 [1]	Bio_CH	-	Verified
#14	FE_ID 9[1]	CSIC	-	Verified
#15	PEH_Pbau_PE-H[2]	BIOSYNTH	03917623SS0925	Verified
#16	PEH_Poce_PE-H[2]	BIOSYNTH	03919323SS1115	Verified

[1]The enzymes in the table are ordered by a number #XX, which corresponds to their order of appearance in the "Enzyme Specification Sheets" detailed below.

[2]In Supplementary Material are shown the results for PEH_Pbau_PE-H (Suplemmentary Fig. 4) and PEH_Poce_PE-H (Supplementary Fig. 4-6).

*Activity with model substrates

Enzyme specification sheet

Original version:	03.11.2023	UDUS	Stephan Thies Rebecka Molitor	s.thies@fz-juelich.de r.molitor@fz-juelich.de
Last update:	[date]	[institution]	[Name]	[email]

Enzyme:	**#01**	**Dim_#008**

Enzyme class:	List of enyzme preparations (quantities for industrial tests have already been shared with partners):	Target application sector(s):
Esterase / Lipase	1. Batch 03914023SC0824 | Biosynth] | EstLip-Dim#008	☒ Detergents ☒ Textiles (specify) ☒ Oil removal ☐ Dye removal ☐ Polyester end of life ☐ Cosmetics ☐ Other:_____

Enzyme origin

Identifying partner:	UDUS	Type of expression:	Intracellular	Mode of application:	Soluble enzyme
Original host:	*E.coli*	Purification and formulation:	Other (specify) Purified from cells, stored in buffer Extract	Measured activity [unit]:	Olive oil, 43.5 U/mg (https://www.sciencedirect.com/science/article/pii/S135951131200387X?via%3Dihub
Further characteristics / comments:	C-term His-Tag for purification				

Production batch: 03914023SC0824

Producing partner:	Biosynth	Type of expression:	Secreted	Mode of application:	Soluble enzyme
Production host:	*Pichia pastoris*	Purification and formulation:	Lyophylised Supernatant	Measured activity:	pNP-Butyrate
Further characteristics / comments:	Material shows comparably low enzyme content (Annex Fig. 1)				

Production batch: [Batch number]

Producing partner:	[institution]	Type of expression:	Choose	Mode of application:	Choose
Production host:	[host organism]	Purification and formulation:	[formulation] [enyzme fraction]	Measured activity:	[text]
Further characteristics / comments:	[text]				

Application 1: Textiles (Oil removal)

Recommended application conditions (from research perspective):			
Temperature [°C]*:	30°C or 40°C	Substrate:	61488Z ROH
pH [-]*:	7-8	Substrate concentration [unit]: [description of the unit]	Ca. 8 mg fabric/100µl reaction = 80 g fabric/L
Medium (water, buffer, salt, cofactors, etc.)*:	0.1 M Phosphate buffer, Surfactant: 1% Triton -X	Expected product(s) and their quantities:	Expected products: Fatty acids Measured products: free long chain fatty acids
Time / duration:	2 h (see Annex Fig. 2)	Analytical methods:	NEFA free long chain fatty acid detection Kit
Enzyme concentration [unit]: [description of the unit]	0.3-1.8 g/L lyophilised material Good results already with 0.3 g/L (Annex Fig. 2) =ca. 3.75mg powder/g fabric	Other remarks on the set-up of the enzyme reaction:	Activity expected towards: Triglycerides, wax esters, esters in general
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

Brief description of observed activity (under recommended conditions):	See Annex Fig. 2
Detailed methodology of activity assay:	- Given amounts of enzymes refer to mg material per reaction - Schoeller-fabric Nr. 61488Z ROH was chosen because it lead to the clearest signals in previous experiments) - Triton-X as supplement to solubilize long chain fatty acids - Method: Incubation of enzyme + 100 µL buffer + fabric in MTP-wells at 30°C + free long chain fatty acid quantification in the reaction solution using NEFA-KIT To check for buffer effects, we determined fatty acid release after 2h of incubation also with CSIC 's buffer.
Verified positive and negative controls for the reaction:	- Negative control: fabric in Buffer+ Triton-X only.

Tested range of conditions:			
Temperature [°C]:	Melting Point: 65°C (=max-temperature)	Substrate:	Tributyrin, Olive oil, coconut oil , Palmityl palmitate, dibutyl sebacate, Polycaprolactone (530Da), Fabric sample2 ROH, (barely any release of fatty acids from the other fabric samples), Further whole cell extract substrate profile in 10.1128/AEM.00106-20 supplement, Designation CE01
pH [-]:	4-11 (https://www.sciencedirect.com/science/article/pii/S1359511312003 87X?via%3Dihub)	Substrate concentration [unit]:	1-10 mM (= up to ca. 8 g/L) 80 g/L for fabrics
Medium (water, buffer, salt, cofactors, etc.):	0.1 M Phosphate buffer 40 mM HEPES buffer+150mM salt,	Expected product(s) and their quantities:	Fatty acids, dicarboxylic acids

	if applicable: Surfactant: 1% Triton – X, Mg-ions: decrease of activity, Ca-ions: Beneficial for activity https://www.sciencedirect.com/science/article/pii/S135951131200387X?via%3Dihub)		
Time / duration:	0.5 h-24 h	Analytical methods:	NEFA free long chain fatty acid detection Kit, acidification,
Enzyme concentration [unit]:	0.3-1.8 g/L lyophilised material	Other remarks:	[text]

Application 2: Detergents

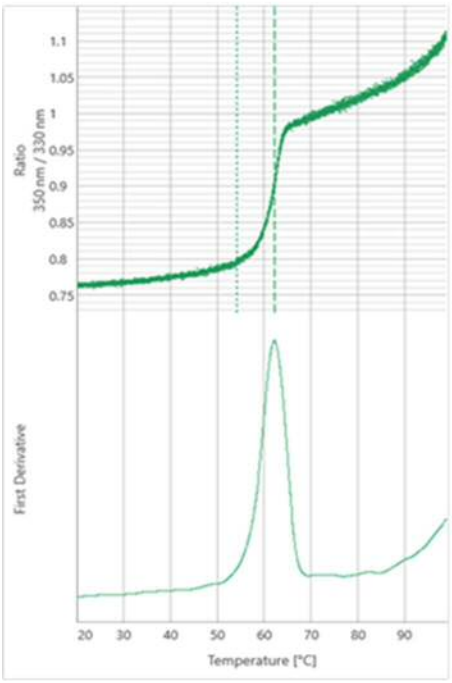
Recommended application conditions (from research perspective):			
Temperature [°C]*:	40°C	Substrate:	[PC-S-16 Lipstick, pink, Material: Polyester/Cotton (PCN-01), PC-S-61B Beef fat colored with violet dye, Material: Polyester/Cotton (PCN-01),
pH [-]*:	7-8	Substrate concentration [unit]: [description of the unit]	8 mg fabric/reaction vessel = 80 g fabric/L
Medium (water, buffer, salt, cofactors, etc.)*:	0.1 M Phosphate buffer, Surfactant: 1% Triton -X	Expected product(s) and their quantities:	Expected products: Fatty acids Measured products: free long chain fatty acids
Time / duration:	2 h (see Annex Fig. 3)	Analytical methods:	NEFA free long chain fatty acid detection Kit
Enzyme concentration [unit]: [description of the unit]	0.2 g/L & 0.5g/L lyophilised material Good results already with 0.2 g/L	Other remarks on the set-up of the enzyme reaction:	- Activity expected towards: Triglycerides, wax esters, esters in general - Not stable in Henkel washing liquor
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

Brief description of observed activity (under recommended conditions):	See Annex Fig. 3
Detailed methodology of activity assay:	- Given amounts of enzymes refer to mg material per reaction - Triton-X as supplement to solubilize long chain fatty acids - Method: Incubation of enzyme + 100 µL buffer + fabric in MTP-wells at 30°C + free long chain fatty acid quantification in the reaction solution using NEFA-KIT
Verified positive and negative controls for the reaction:	- Negative control: fabric in Buffer+ Triton-X only. (positive control for Lipstick stain): Henkel washing liquor+enzymes)

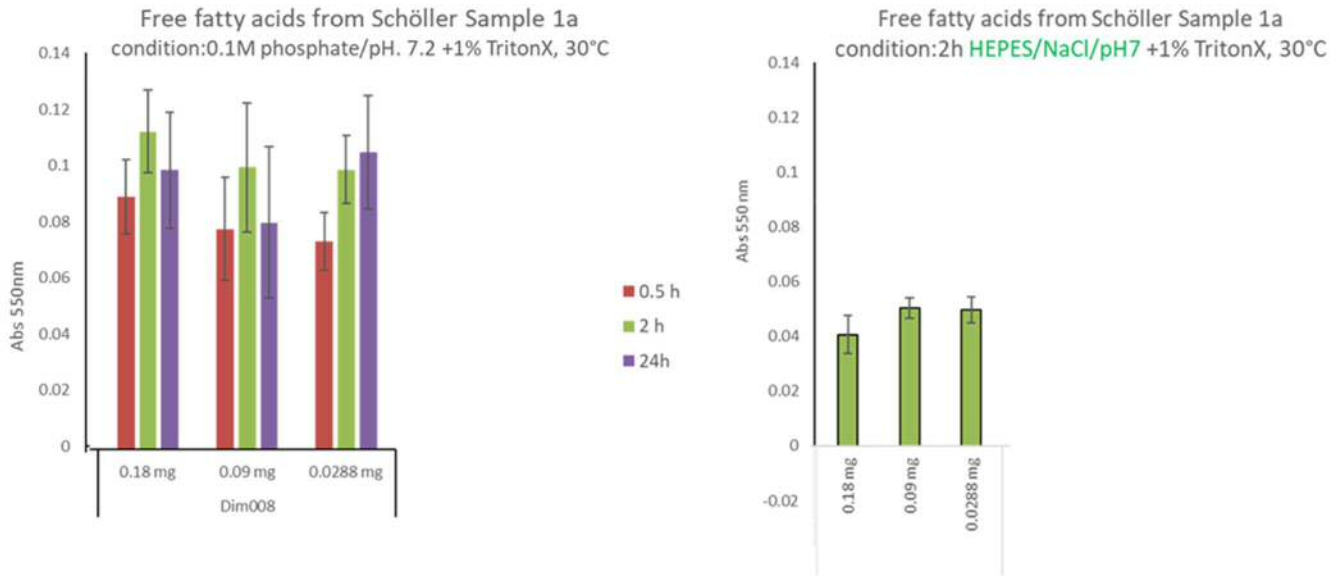
Tested range of conditions:			
Temperature [°C]:	Melting Point:65°C =max-temperature)	Substrate:	Tributyrin, Olive oil, coconut oil , Palmityl palmitate, dibutyl sebacate, Polycaprolactone (530Da), Further whole cell extract substrate profile in 10.1128/AEM.00106-20 supplement, Designation CE01
pH [-]:	4-11 (https://www.sciencedirect.com/science/article/pii/S1359511312003 87X?via%3Dihub)	Substrate concentration [unit]:	1-10mM (= up to ca. 8g/L) 80g/L for fabrics
Medium (water, buffer, salt, cofactors, etc.):	0.1 M Phosphate buffer, 40 mM HEPES buffer+150mM salt,	Expected product(s) and their quantities:	Fatty acids, dicarboxylic acids

	if applicable: Surfactant: 1% Triton – X Clacium		
Time / duration:	0.5h-24h	Analytical methods:	NEFA free long chain fatty acid detection Kit, acidification
Enzyme concentration [unit]:	0.2 g/L & 0.5g/L lyophilised material Good results already with 0.2 g/L	Other remarks:	

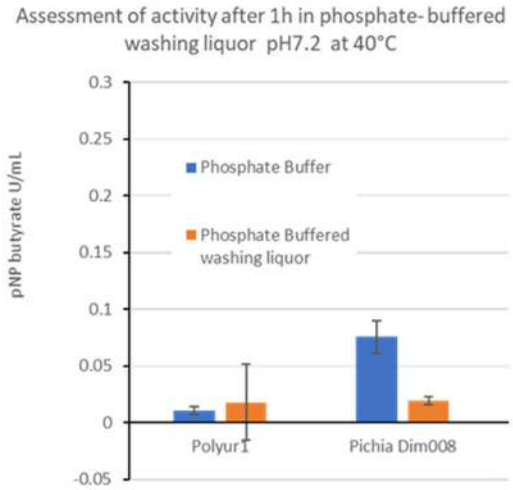
Attachment (additional figures, descriptions, etc.)

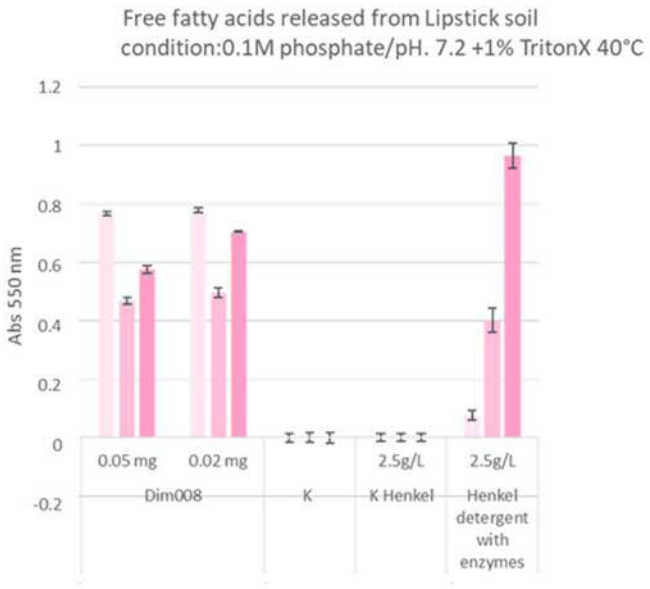


Annex Fig. 1 Left: Melting curves of Biosynth lyophilised material using Differential Scanning fluorometry, indicating a melting point at 65°C. Right: Coomassie stained SDS page of 10mg/mL lyophilised material indicating a rather low enzyme content of this preparation.



Annex Fig. 2. Release of fatty acids from sample 1a (61488Z ROH) in two different conditions (Output: signal intensity at 550nm). Left: 0.1 Potassium phosphate, pH7.2, no salt; right: CSIC Buffer (40 mM HEPES, pH,7, 150 mM salt), each with Triton X as a surfactant to solubilize fatty acids. It confirms activity to the oils on the fabric and further shows decreased activity of this enzyme under the HEPES/pH7/salt conditions. Stated enzyme amount represent the absolute amount of enzyme powder in 100µL reaction





Annex Fig. 3. Dim008 for detergent application. Left: Residual activity in washing liquor indicates engineering demand of this lipase. Right: Fatty acid release from Lipstick-soiled textiles determines after 0.5h, 2h, and 24h indicates activity against fatty stains in the presence of the milder surfactant TritonX. Stated enzyme amount represent the absolute amount of enzyme powder in 100µL reaction.

Enzyme specification sheet

Original version:	06.11.2023	UDUS	Stephan Thies Rebecka Molitor	s.thies@fz-juelich.de r.molitor@fz-juelich.de
Last update:	[date]	[institution]		

Enzyme:	**#02**	**Paes_TB035**

Enzyme class:	List of enyzme preparations (quantities for industrial tests have already been shared with partners):	Target application sector(s):
Esterase / Lipase	2. 03908323SS0521 | Biosynth | EstLip-Paes-TB035	☒ Detergents ☒ Textiles (specify) ☒ Oil removal ☐ Dye removal ☐ Cosmetics ☐ Other:_____

Enzyme origin

Identifying partner:	UDUS	Type of expression:	Intracellular	Mode of application:	Solubilised enzyme
Original host:	*E. coli*	Purification and formulation:	Other (specify) purified from cell extract [enyzme fraction]	Measured activity [unit]:	pNP Butyrate
Further characteristics / comments:	C-term His-Tag for purification				

Production batch: 03908323SS0521

Producing partner:	Biosynth	Type of expression:	Secreted	Mode of application:	Soluble enzyme
Production host:	*Pichia pastoris*	Purification and formulation:	Lyophylised Supernatant	Measured activity:	pNP Butyrate
Further characteristics / comments:					

Production batch: [Batch number]

Producing partner:	[institution]	Type of expression:	Choose	Mode of application:	Choose
Production host:	[host organism]	Purification and formulation:	[formulation] [enyzme fraction]	Measured activity:	[text]
Further characteristics / comments:	[text]				

Application 1: Textiles (Oil removal)

Recommended application conditions (from research perspective):			
Temperature [°C]*:	30°C or 40°C	Substrate:	61488Z ROH
pH [-]*:	Standard:7.2 Optimal:10	Substrate concentration [unit]: [description of the unit]	Ca. 8 mg fabric/100µl reaction = 80 g fabric/L
Medium (water, buffer, salt, cofactors, etc.)*:	0.1 M Phosphate buffer, Surfactant: 1% Triton -X	Expected product(s) and their quantities:	Expected products: Fatty acids Measured products: free long chain fatty acids
Time / duration:	2 h	Analytical methods:	NEFA free fatty acid detection Kit
Enzyme concentration [unit]: [description of the unit]	1.8 g/L lyophilised material =ca. 23mg powder/g fabric	Other remarks on the set-up of the enzyme reaction:	- not a lipase - Activity expected towards: Triglycerides with C4-10 , large variety of short chained and aromatic esters in general
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

Brief description of observed activity (under recommended conditions):	See Annex Fig. 5
Detailed methodology of activity assay:	- Given amounts of enzymes refer to mg material per reaction - Schoeller-fabric Nr. 61488Z ROH was chosen because it lead to the clearest signals in previous experiments) - Triton-X as supplement to solubilize long chain fatty acids - Method: Incubation of enzyme + 100 µL buffer + fabric in MTP-wells at 30°C + free long chain fatty acid quantification in the reaction solution using NEFA-KIT Also: check for fatty acid release after 2h of incubation also with CSIC's buffer.
Verified positive and negative controls for the reaction:	- Negative control: fabric in Buffer+ Triton-X only. - positive control: Dim008

Tested range of conditions:			
Temperature [°C]:	Melting Point:65.2°C =maximal temperature)	Substrate:	Tributyrin, dibutyl sebacate, Polycaprolactone (530Da), Diesters of furandicarboxyl acid and terephthalic acid Fabric sample2 ROH, (barely any release of fatty acids from the other fabric samples), Further whole cell extract substrate profile in 10.1128/AEM.00106-20 supplement, Designation CE013
pH [-]:	6.5-11	Substrate concentration [unit]:	1-10mM (= up to ca. 8g/L)
Medium (water, buffer, salt, cofactors, etc.):	0.1 M Phosphate buffer, 40 mM HEPES buffer+150mM salt 0.1M Tris buffer, MES buffer,	Expected product(s) and their quantities:	Fatty acids, dicarboxylic acids

	if applicable: Surfactant: 1% Triton - X		
Time / duration:	10min-24h	Analytical methods:	NEFA free long chain fatty acid detection Kit, acidification
Enzyme concentration [unit]:	0.6g/L	Other remarks:	

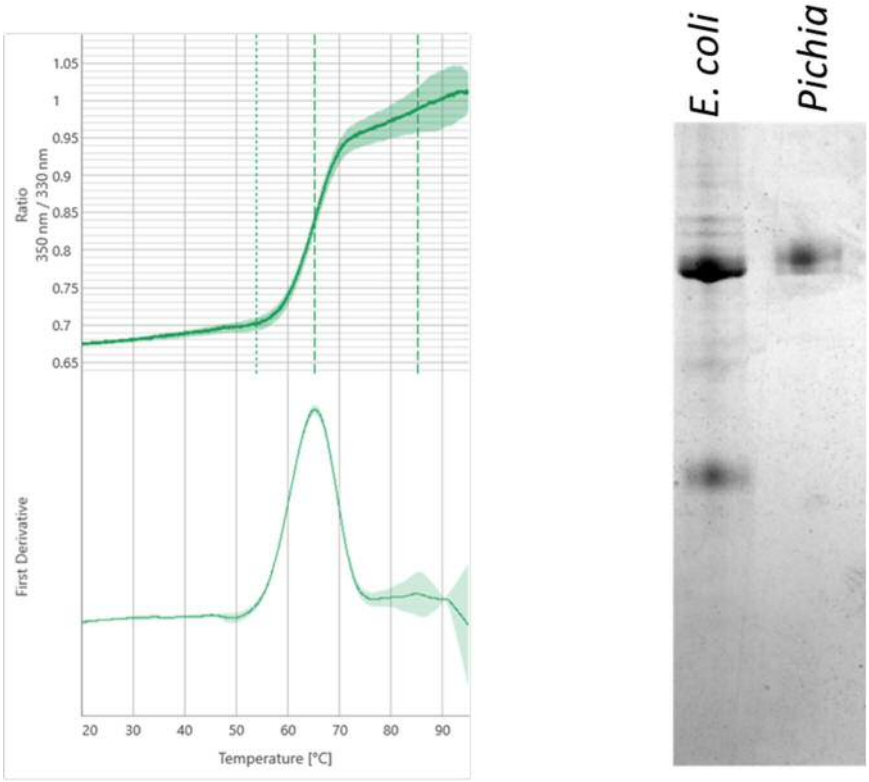
Application 2: Detergents

Recommended application conditions (from research perspective):			
Temperature [°C]*:	30°C or 40°C	Substrate:	Tributyrin, dibutyl sebacate, Polycaprolactone (530Da), No experiments with standard-soiled textiles
pH [-]*:	Standard:7.2 Optimal:10	Substrate concentration [unit]: [description of the unit]	1-10mM (= up to ca. 8g/L)
Medium (water, buffer, salt, cofactors, etc.)*:	0.1 M Phosphate buffer, Surfactant: 1% Triton -X	Expected product(s) and their quantities:	Fatty acids, dicarboxylic acids
Time / duration:	2 h	Analytical methods:	NEFA free long chain fatty acid detection Kit, acidification
Enzyme concentration [unit]: [description of the unit]	0.6g/L	Other remarks on the set-up of the enzyme reaction:	- Exceptionally stable in any tested detergent, including Henkel washing liquor - not a lipase - Activity expected towards: Triglycerides with C4-10 , large variety of short chained and aromatic esters in general
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

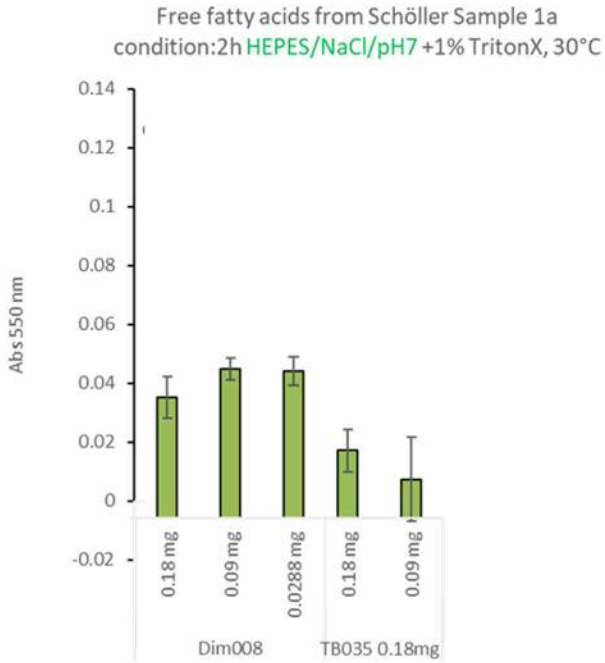
Brief description of observed activity (under recommended conditions):	See Annex Fig. 6
Detailed methodology of activity assay:	- Given amounts of enzymes refer to mg material per reaction - Triton-X as s supplement to solubilize long chain fatty acids - Method: Incubation of enzyme + 100 µL buffer + fabric in MTP-wells at 30°C + free long chain fatty acid quantification in the reaction solution using NEFA-KIT Also: check for fatty acid release after 2h of incubation CSIC's buffer
Verified positive and negative controls for the reaction:	Negative: buffer without enzyme

Tested range of conditions:			
Temperature [°C]:	Melting Point:65.2°C =maximal temperature)	Substrate:	Further whole cell extract substrate profile in 10.1128/AEM.00106-20 supplement, Designation CE013
pH [-]:	6.5-11	Substrate concentration [unit]:	1-10mM (= up to ca. 8g/L)
Medium (water, buffer, salt, cofactors, etc.):	0.1 M Phosphate buffer, 40 mM HEPES buffer+150mM salt 0.1M Tris buffer, MES buffer, if applicable: Surfactant: 1% Triton -X	Expected product(s) and their quantities:	Fatty acids, dicarboxylic acids
Time / duration:	10min-24h	Analytical methods:	NEFA free long chain fatty acid detection Kit, acidification
Enzyme concentration [unit]:	0.6g/L	Other remarks:	

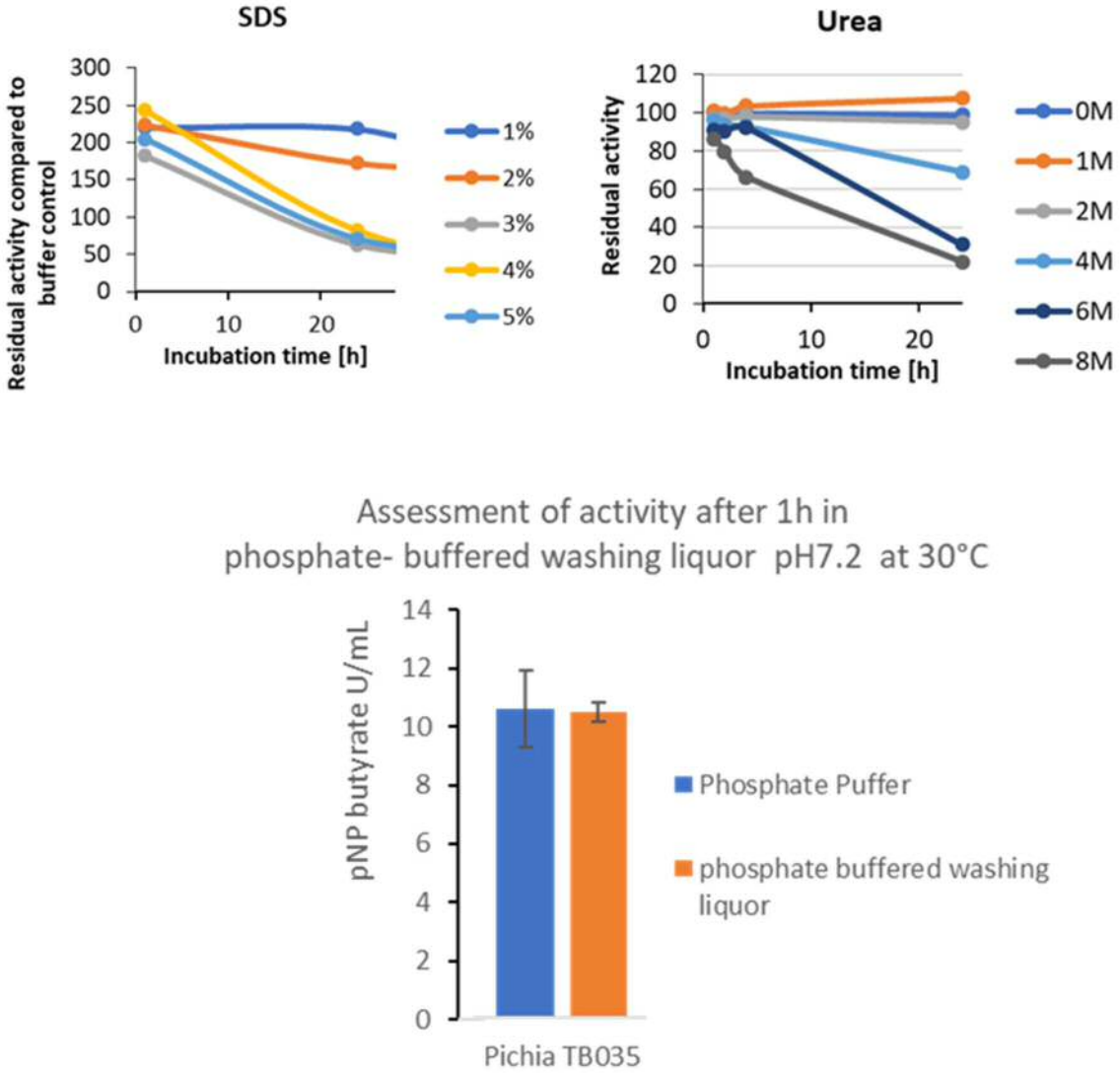
Attachment (additional figures, descriptions, etc.)



Annex Fig. 4. Left: Melting curves of Biosynth lyophilised material using Differential Scanning fluorometry, indicating a melting point at 65°C. Right: Coomassie stained SDS page to compare **1mg/mL** lyophilised material and the E. coli preparation. The absence of the lower band that consists of a globular protein fraction not unfolded by SDS in the Pichia-derived protein sample might indicate an increased susceptibility to harsh surfactants.



Annex Fig. 5. Release of fatty acids from sample 1a (61488Z ROH) in CSIC Buffer (40 mM HEPES, pH,7, 150 mM salt) with Triton X as a surfactant to solubilize fatty acids. It confirms minor activity towards the oils on the fabric. Stated enzyme amounts represent the absolute amount of enzyme powder in 100µL reaction.



Annex Fig. 6. Resistance of TB035 to detergents. Upper row: resistance towards denaturing agents SDS (anionic detergent) and urea (chaotropic salt. These experiments were done with E coli preparations Lower row, left: Residual activity in washing liquor (0.1 g/L enzyme).

Additional information is shown in Supplementary Material (Supplementary Fig. 3).

Enzyme specification sheet

Original version:	16.01.2024	UDUS	Rebecka Molitor	r.molitor@fz-juelich.de
Last update:	18.01.2024	UDUS	Rebecka Molitor	r.molitor@fz-juelich.de

Enzyme:	**#03**	**PEH-Pform_PE-H (Biosynth preparation)**	
Enzyme class:	List of enyzme preparations (quantities for industrial tests have already been shared with partners):		Target application sector(s):
PETase	1. 03917823SS1013 | Biosynth | Pform_PE-H		☒ Detergents ☒ Textiles (specify) ☒ Oil removal ☐ Dye removal ☐ Cosmetics ☐ Other:_____

Enzyme origin					
Identifying partner:	UDUS	Type of expression:	Intracellular	Mode of application:	Solubilised enzyme
Original host:	*E. coli*	Purification and formulation:	Other: Purified from cells, stored in buffer .	Measured activity [unit]:	[text]
Further characteristics / comments:	[text]				

Production batch: 03917823SS1013					
Producing partner:	Biosynth	Type of expression:	Secreted	Mode of application:	Choose
Production host:	*Pichia pastoris*	Purification and formulation:	Lyophylised whole cells	Measured activity:	[text]
Further characteristics / comments:	[text]				

Production batch: **[Batch number]**					
Producing partner:	[institution]	Type of expression:	Choose	Mode of application:	Choose
Production host:	[host organism]	Purification and formulation:	[formulation] [enyzme fraction]	Measured activity:	[text]
Further characteristics / comments:	[text]				

Application 1: Detergents

Recommended application conditions (from research perspective):			
Temperature [°C]*:	40°C	Substrate:	Beef fat and lipstick
pH [-]*:	7-8	Substrate concentration [g/L]: [g_{fabric}/L_{buffer}]	80 g/L
Medium (water, buffer, salt, cofactors, etc.)*:	0.1 M Phosphate buffer, Surfactant: 1% Triton -X	Expected product(s) and their quantities:	Expected products: Fatty acids Measured products: free long chain fatty acids
Time / duration:	24 h	Analytical methods:	NEFA free long chain fatty acid detection Kit,
Enzyme concentration [mg] [$mg_{lyophilised\ material\ per\ reaction}$]	0.05 mg	Other remarks on the set-up of the enzyme reaction:	- stable in Henkel washing liquor Activity expected towards: Triglycerides, wax esters, esters in general, Polyester
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

Brief description of observed activity (under recommended conditions):	- Enzyme was not visible on SDS-PAGE but did show significant activity at the recommended conditions (Annex Fig. 7) - 0.05 mg enzyme in Kpi buffer showed activity comparable to positive control after 24 h on beef fat (Annex Fig. 8) - 0.05 mg enzyme in Kpi buffer showed activity comparable to positive control after 24 h on lipstick (Annex Fig. 9)
Detailed methodology of activity assay:	- Given amounts of enzymes refer to mg material per reaction. - Substrates beef fat and lipstick - Triton-X as supplement to solubilize long chain fatty acids. - Method: Incubation of enzyme + 100 µL buffer + fabric in MTP-wells at 40°C + free long chain fatty acid quantification in the reaction solution using NEFA-KIT - To check for buffer effects, we determined fatty acid release also with CSIC 's buffer.
Verified positive and negative controls for the reaction:	- Negative control: fabric in buffer+ Triton-X only, fabric in buffer with detergent without enzymes - Positive control: oleic acid in buffer, fabric with detergent with enzymes

Tested range of conditions:			
Temperature [°C]:	Melting Point measured for *E. coli*-produced Enzyme: 53.9°C (preliminary due to broken nanoDSF device)	Substrate:	Beef fat and lipstick standard pollution
pH [-]:	7-8	Substrate concentration [g/L]:	ca. 8 g/L
Medium (water, buffer, salt, cofactors, etc.):	0.1 M Phosphate buffer 40 mM HEPES buffer+150mM salt,	Expected product(s) and their quantities:	Fatty acids, dicarboxylic acids

	if applicable: Surfactant: 1% Triton - X		
Time / duration:	0.5h-24h	Analytical methods:	NEFA free long chain fatty acid detection Kit, acidification,
Enzyme concentration [mg]	0.05-0.02 mg	Other remarks:	[text]

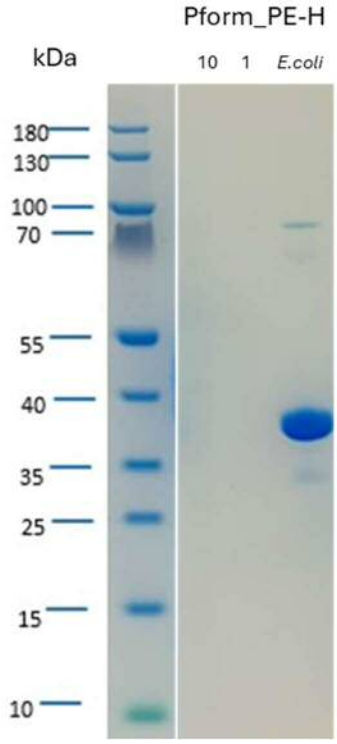
Application 2: Textiles (Oil removal)

Recommended application conditions (from research perspective):			
Temperature [°C]*:	40°C	Substrate:	61488Z ROH (barely any release of fatty acids from the other fabric samples)
pH [-]*:	7.2	Substrate concentration [g/L] [g_{fabric}/L_{buffer}]	80 g/L
Medium (water, buffer, salt, cofactors, etc.)*:	0.1 M Phosphate buffer, Surfactant: 1% Triton -X	Expected product(s) and their quantities:	Expected products: Fatty acids Measured products: free long chain fatty acids
Time / duration:	24 h	Analytical methods:	NEFA free long chain fatty acid detection Kit,
Enzyme concentration [mg]: [mg enzyme per reaction]	0.18 mg	Other remarks on the set-up of the enzyme reaction:	Activity expected towards: Triglycerides, wax esters, esters in general, Polyester
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

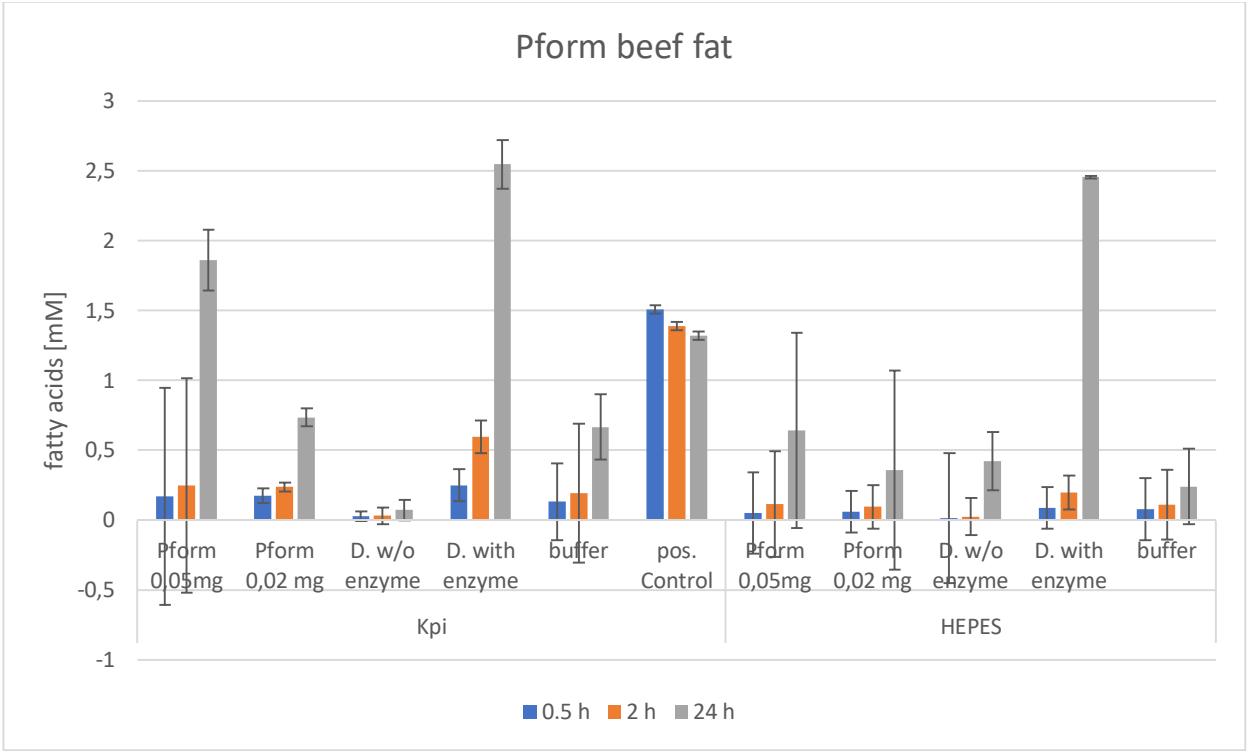
Brief description of observed activity (under recommended conditions):	- Enzyme was not visible on SDS-PAGE but did show significant activity at the recommended conditions (Annex Fig. 7) - 0.18 mg of enzyme solution showed minor activity after 24 h (Annex Fig. 10)
Detailed methodology of activity assay:	- Given amounts of enzymes refer to mg material per reaction - Schoeller-fabric Nr. 61488Z ROH was chosen because it lead to the clearest signals in previous experiments - Triton-X as supplement to solubilize long chain fatty acids - Method: Incubation of enzyme + 100 µL buffer + fabric in MTP-wells at 40°C + free long chain fatty acid quantification in the reaction solution using NEFA-KIT - To check for buffer effects, we determined fatty acid release also with CSIC 's buffer (HEPES).
Verified positive and negative controls for the reaction:	- Positive control: oleic acid in buffer - Negative control: buffer without enzyme

Tested range of conditions:			
Temperature [°C]:	40°C	Substrate:	61488Z ROH (barely any release of fatty acids from the other fabric samples)
pH [-]:	7-8	Substrate concentration [g/L]:	80 g/L
Medium (water, buffer, salt, cofactors, etc.):	0.1 M Phosphate buffer 40 mM HEPES buffer+150mM salt, if applicable: Surfactant: 1% Triton -X	Expected product(s) and their quantities:	Fatty acids, dicarboxylic acids
Time / duration:	0.5 h-24 h	Analytical methods:	NEFA free long chain fatty acid detection Kit, acidification,
Enzyme concentration [mg]:	0.0288-0.18 mg	Other remarks:	[text]

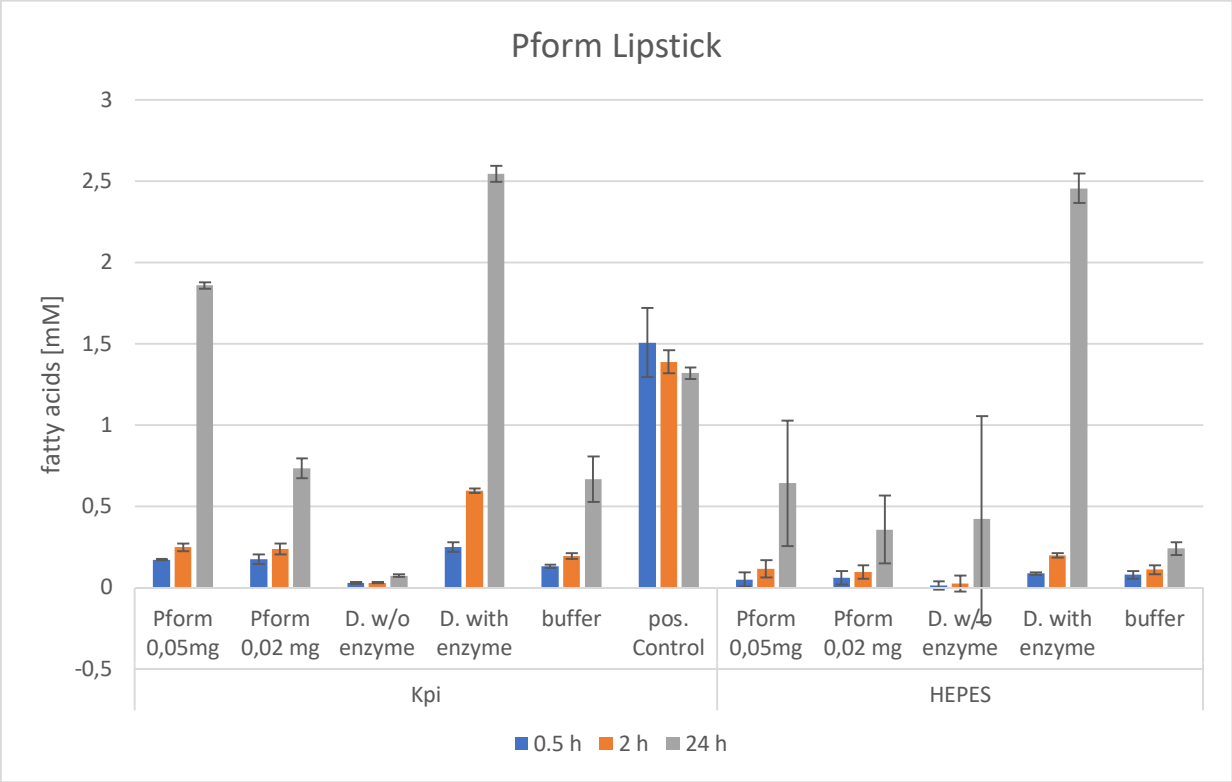
Attachment (additional figures, descriptions, etc.)



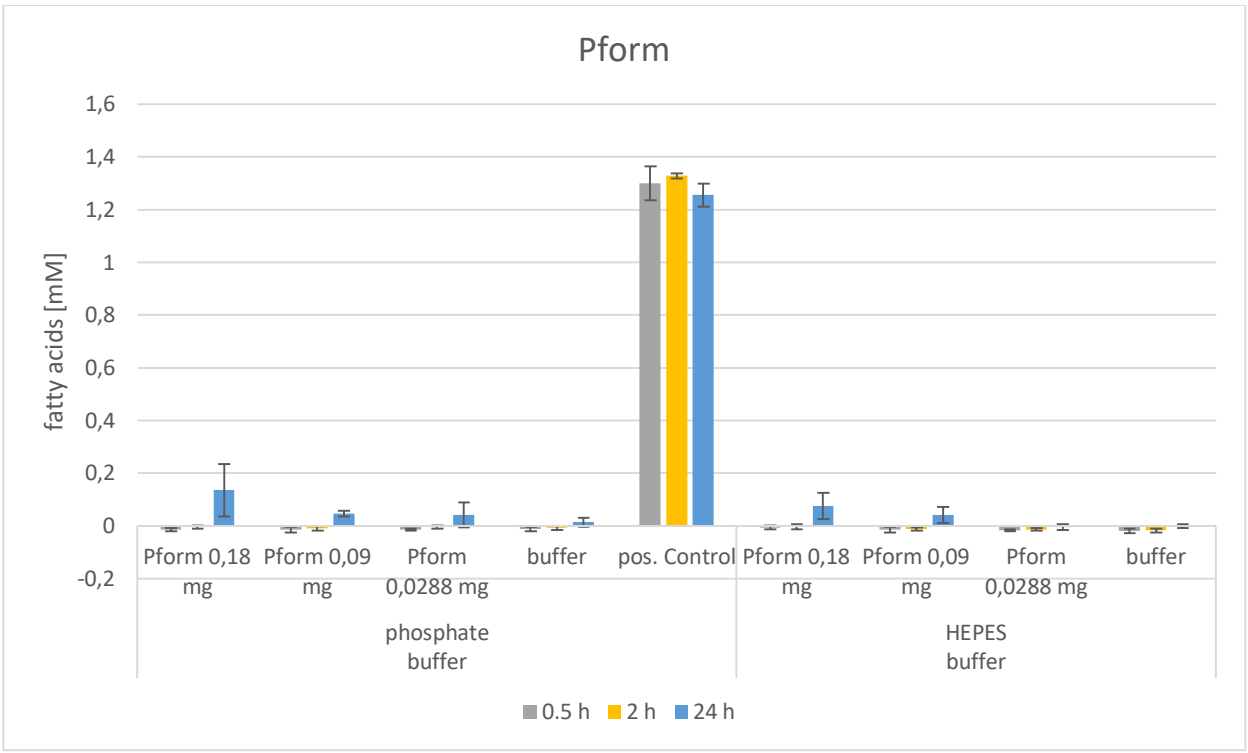
Annex Fig. 7. SDS-PAGE of Pichia pastoris and E. coli enzymes, concentrations of 10 mg/ml and 1 mg/ml (Pichia), E. coli produced enzyme undiluted as reference. 5 µl applied.



Annex Fig. 8. Pform_PE-H tested with beef fat standard pollution incubated in HEPES and Kpi buffer between 0.5 and 24 h. The enzyme was tested in two different concentrations and with the addition of detergent (D.). as negative controls the detergent was tested without enzymes as well as only the buffer.



Annex Fig. 9. Pform_PE-H tested with lipstick standard pollution incubated in HEPES and Kpi buffer between 0.5 and 24 h. The enzyme was tested in two different concentrations and with the addition of detergent (D.). as negative controls the detergent was tested without enzymes as well as only the buffer.



Annex Fig. 10. Pform_PE-H tested with the substrate textile 61488Z ROH for 0.5-24 h at 40°C in Kpi buffer and HEPES buffer.

Additional information is shown in Supplementary Material (Supplementary Fig. 4).

Enzyme specification sheet

Original version:	[date]19.01.2024 14:51:00	BANGOR	[Name]	a.khusnutdinova@bangor.ac.uk
Last update:	19.01.2024	BANGOR	[Name]	a.khusnutdinova@bangor.ac.uk

Enzyme:	**#04**	**[GEN0105]**	
Enzyme class:	List of enyzme preparations (quantities for industrial tests have already been shared with partners):		Target application sector(s):
choose or enter	Batch 03920023SS1120		☒ Detergents ☐ Textiles (specify) ☐ Oil removal ☐ Dye removal ☐ Cosmetics ☐ Other:_____

Enzyme origin					
Identifying partner:	BANGOR	Type of expression:	Intracellular	Mode of application:	Solubilised enzyme
Original host:	Metagenome library from Evry mesophilic anaerobic digester	Purification and formulation:	Cell-free (liquid) His-tag purified	Measured activity [unit]:	V_{max} = 11.7±1.2 [U/mg], k_{cat}=7.0±0.7 [s^{-1}], k_{cat}/K_m=0.8x10^4 [$M^{-1}s^{-1}$]
Further characteristics / comments:	Activity measured with pNp-hexanoate, in TrisCl 50mM pH 8.0 buffer, reaction detected in 96 well plate at 410 nm, reaction volume 200ul				

Production batch: **[03920023SS1120]**					
Producing partner:	Biosynth	Type of expression:	Choose	Mode of application:	Other (specify)
Production host:	*P. pastoris*	Purification and formulation:	[formulation] [enyzme fraction]	Measured activity:	V_{max} = 0.45±0.035[U/mg], k_{cat}=0.27±0.2 [s^{-1}], k_{cat}/K_m=(1.7±0.13)10^3 [$M^{-1}s^{-1}$]
Further characteristics / comments:	Activity measured with pNp-hexanoate, in TrisCl 50mM pH 8.0 buffer, reaction detected in 96 well plate at 410 nm, reaction volume 200ul				

Production batch: **[Batch number]**					
Producing partner:	[institution]	Type of expression:	Choose	Mode of application:	Choose
Production host:	[host organism]	Purification and formulation:	[formulation] [enyzme fraction]	Measured activity:	[text]
Further characteristics / comments:	[text]				

Application 1: Detergents

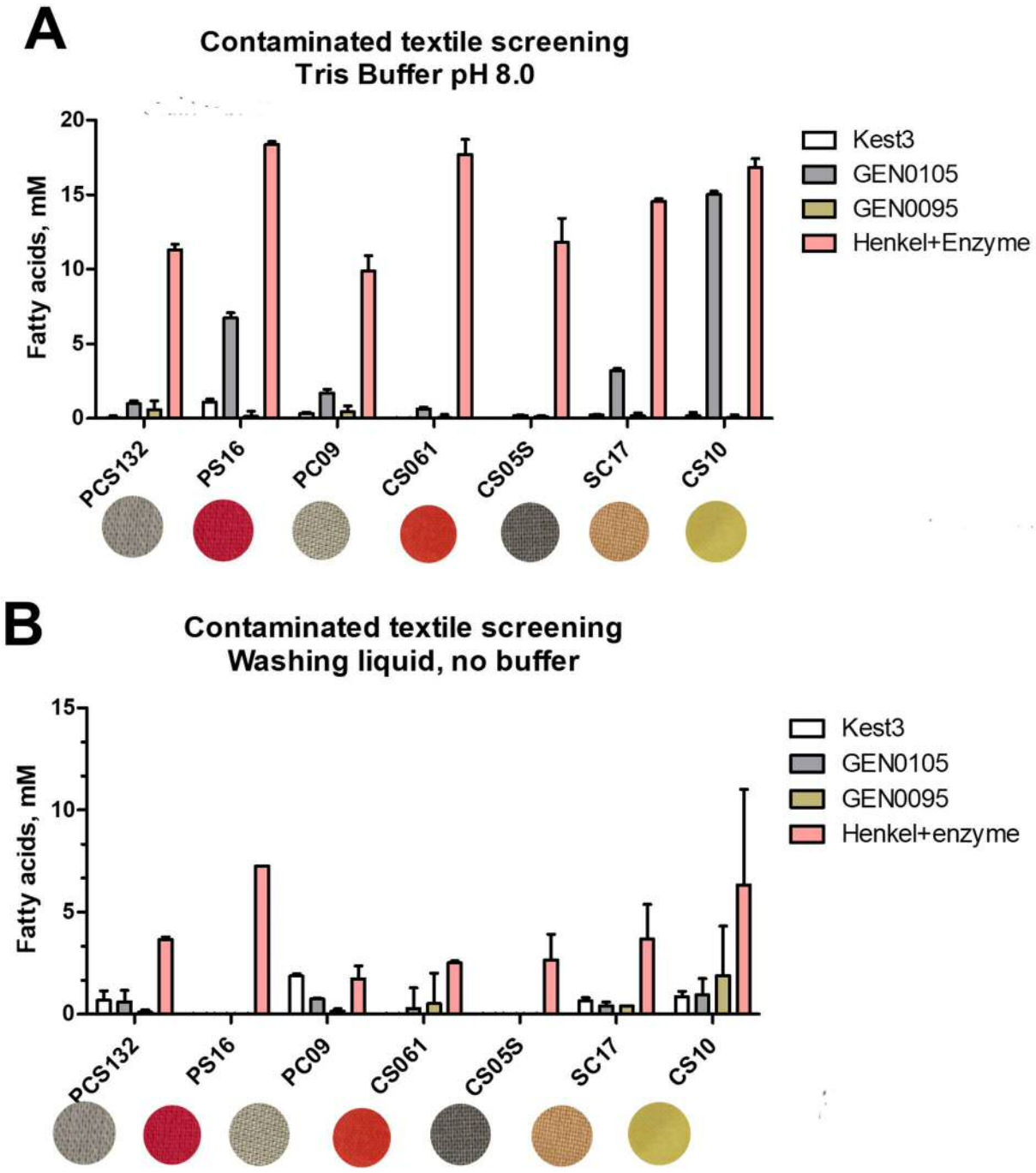
Recommended application conditions (from research perspective):			
Temperature [°C]*:	[30-50°C (T_m=43.7°C, but in short term max product is observed at 50°C)]	Substrate:	[Tested with C_2-C_{14}-pNp substrate, highest activity observed with pNp-butyrate. Tested activity with plastic (amorphousPET, 3PET, PCL and PDLA), active with all of them. Screened with samples of contaminated textile provided]
pH [-]*:	[8-10]	Substrate concentration [unit]: [description of the unit]	[text]
Medium (water, buffer, salt, cofactors, etc.)*:	[optimal salinity for catalysis is 0.1M NaCl, buffer(used TrisCl, pH 8-10, detergent (Tween20 at 0.5% in reaction mixture increases activity 7.5fold. Reaction volume 200ul]	Expected product(s) and their quantities:	[with 3PET produces mixture of MHET and terephtalic and benzoic acid. With CS10 textile(butterfat on cotton) releases max fatty acids. Washing test released up to 15mM of fatty acids in buffered conditions in presence of Henkel detergent. Not buffered wash released 0.9mM fatty acids from CS10]
Time / duration:	[Henkel textile samples and plastic suspensions reaction was setup for ON=12h]	Analytical methods:	[Henkel textile samples wash test was analysed by NEFA kit, Plastic degradation (PET,PCL,PDLA -HPLC product analysis, pNp-substrates, spectrophotometric read]
Enzyme concentration [ug protein/ml reaction]: [description of the unit]	[0.5-50ug/ml]	Other remarks on the set-up of the enzyme reaction:	[text]
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

Brief description of observed activity (under recommended conditions):	[In buffered conditions and optimal pH can release up to 15mM fatty acids from CS10 sample textile, only water conditions decrease release fatty acids concentrations to 0.96mM. Enzyme showed up to 0.3mM fatty acids release on 4a and 4b pure textiles, that confirmed its activity with ester plastics (Annex Fig. 15-16)
Detailed methodology of activity assay:	[text]
Verified positive and negative controls for the reaction:	[text]

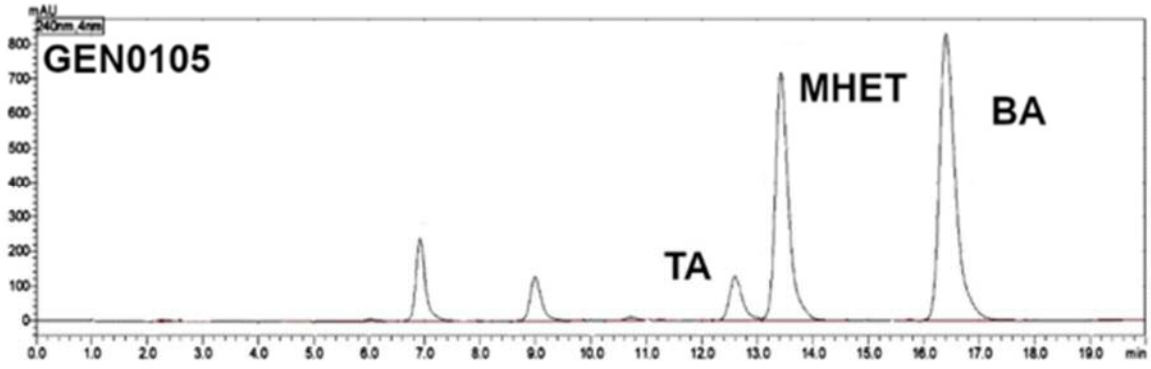
Tested range of conditions:			
Temperature [°C]:	[30-90C]	Substrate:	[pNp-fatty acids, contaminated textile samples, PET,PCL,PDLA plastic suspensions]
pH [-]:	[3-10.5]	Substrate concentration [unit]:	[for C6,C8,C10-pNp substrates were tested 4-0.004mM, textile

			2x2mm size were used for wash in 200ul, 0.125% plastic suspensions were used for test]
Medium (water, buffer, salt, cofactors, etc.):	[NaCl concentration 0-2M, Tween20 0-4.5%]	Expected product(s) and their quantities:	[text]
Time / duration:	[pNp-esters were screening durin 20min incubation, textile and plastic suspension during ON incubation=12h]	Analytical methods:	[Henkel textile samples wash test was analysed by NEFA kit, Plastic degradation (PET,PCL,PDLA -HPLC product analysis, pNp-substrates, spectrophotometric read]
Enzyme concentration [ug/ml]:	[0.5-50ug/ml]	Other remarks:	[text]

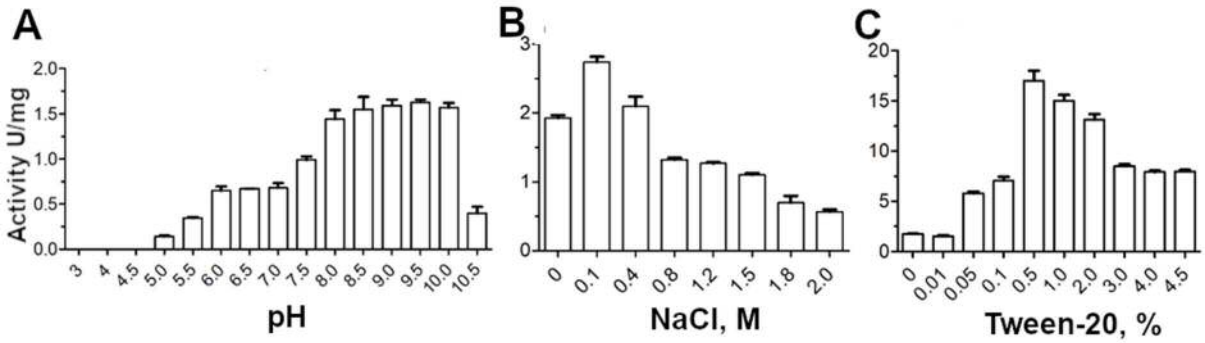
Attachment (additional figures, descriptions, etc.)



Annex Fig. 11. Fabric sample overnight wash with Henkel detergent 3.4g/L, 30C, 500rpm, 50ug enzyme/ml of reaction mixture. 2x2mm textile washed in 200ul reaction volume (A) Reaction set up in 50mM TrisCl buffer pH 8.0 (B) Reaction set up in water.

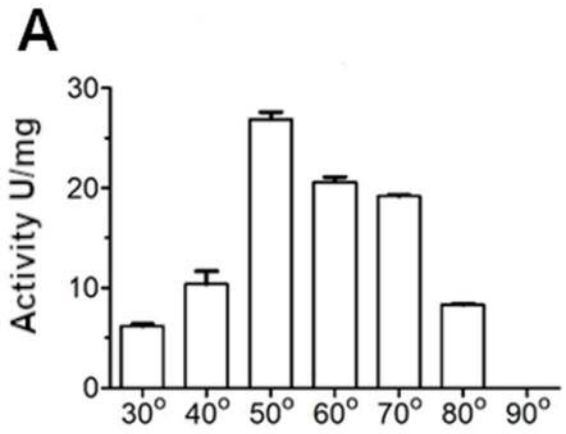


Annex Fig. 12. HPLC analysis of reaction products of 3PET degradation by Gen0105. The reaction mixtures with emulsified 3PET (0.125%) and indicated enzymes (50 µg/ml) were incubated at 30 ºC for 12 h, and reaction products were separated using reverse-phase hydrophobic chromatography on a C18 column. TA=terephtalic acid, BA=benzoic acid

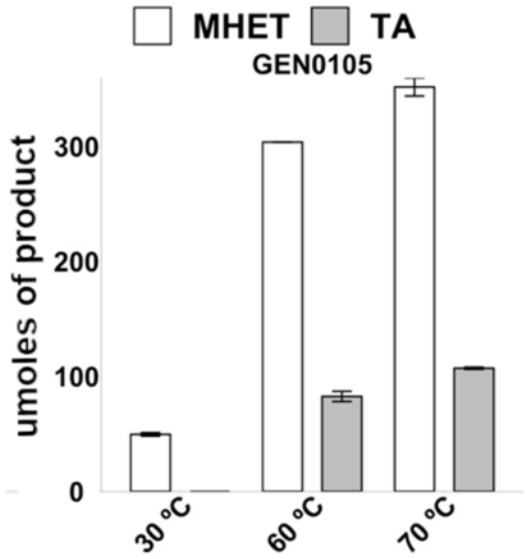


Effect of NaCl and Tween 20 on carboxylesterase activity of purified polyesterases

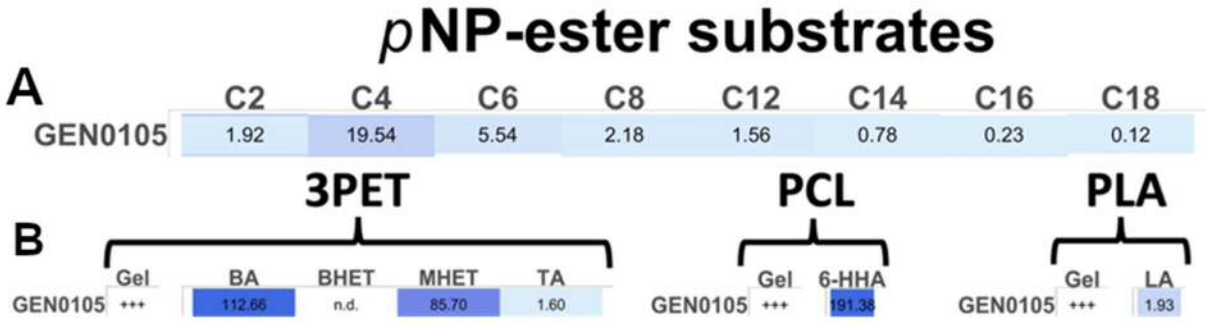
Annex Fig. 13. Effect of pH (A), NaCl(B) and Tween20(C) on carboxylesterase activity of Gen0105 with 1 mM *p*NP-octanoate. The activity was measured using1 µg of enzyme/reaction (20 min incubation at 30ºC) in 200ul reaction mixure.



Annex Fig. 14. Effect of assay temperature on carboxylesterase activity of Gen0105. The reaction mixtures (0.2 ml, 1 µg of enzyme) were incubated at the indicated temperatures for 20 min, and produced p-nitrophenol was measured at 410 nm. As substrates, 1 mM pNP-octanoate was used for reaction.



Annex Fig. 15. Gen0105 amorphous PET plastic degradation activity in emultion 0.125%. (100ug/ml enzyme, 12h incubation at 30 ºC, 60 ºC and 70 ºC, 500rpm)



Annex Fig. 16. Gen0105 pNp-ester substrates (A) and plastic emulsions (B) activity screening. pNp-esters carboxylesterase activity is presented in U/mg, with plastic emultions (B) in mU/mg.

Additional information is shown in Supplementary Material (Supplementary Fig. 4).

Enzyme specification sheet

Original version:	[date]25.01.2024 15:15:00	BANGOR	[Anna Khusnutdinova]	[a.khusnutdinova@bangor.ac.uk]
Last update:	25.01.2024	BANGOR	[Anna Khusnutdinova]	[email]

Enzyme:	**#05**	**[pVec11]**
Enzyme class:	List of enyzme preparations (quantities for industrial tests have already been shared with partners):	Target application sector(s):
choose or enter		☐ Detergents ☐ Textiles (specify) ☐ Oil removal ☒ Dye removal ☐ Cosmetics ☐ Other:_____

Enzyme origin					
Identifying partner:	BANGOR	Type of expression:	Intracellular	Mode of application:	Solubilised enzyme
Original host:	[metagenome library clone expressed in E.coli BL21 Lobstr]	Purification and formulation:	[formulation] Other (Ni-affinity purification)	Measured activity [U/mg]:	[V_{max}=3.5±0.22U/mg, K_m=0.25±0.02 mM] measured for ABTS substrate
Further characteristics / comments:	[text]				

Production batch: [Batch number]					
Producing partner:	[institution]	Type of expression:	Choose	Mode of application:	Choose
Production host:	[host organism]	Purification and formulation:	[formulation] [enyzme fraction]	Measured activity:	[text]
Further characteristics / comments:	[text]				

Production batch: [Batch number]					
Producing partner:	[institution]	Type of expression:	Choose	Mode of application:	Choose
Production host:	[host organism]	Purification and formulation:	[formulation] [enyzme fraction]	Measured activity:	[text]
Further characteristics / comments:	[text]				

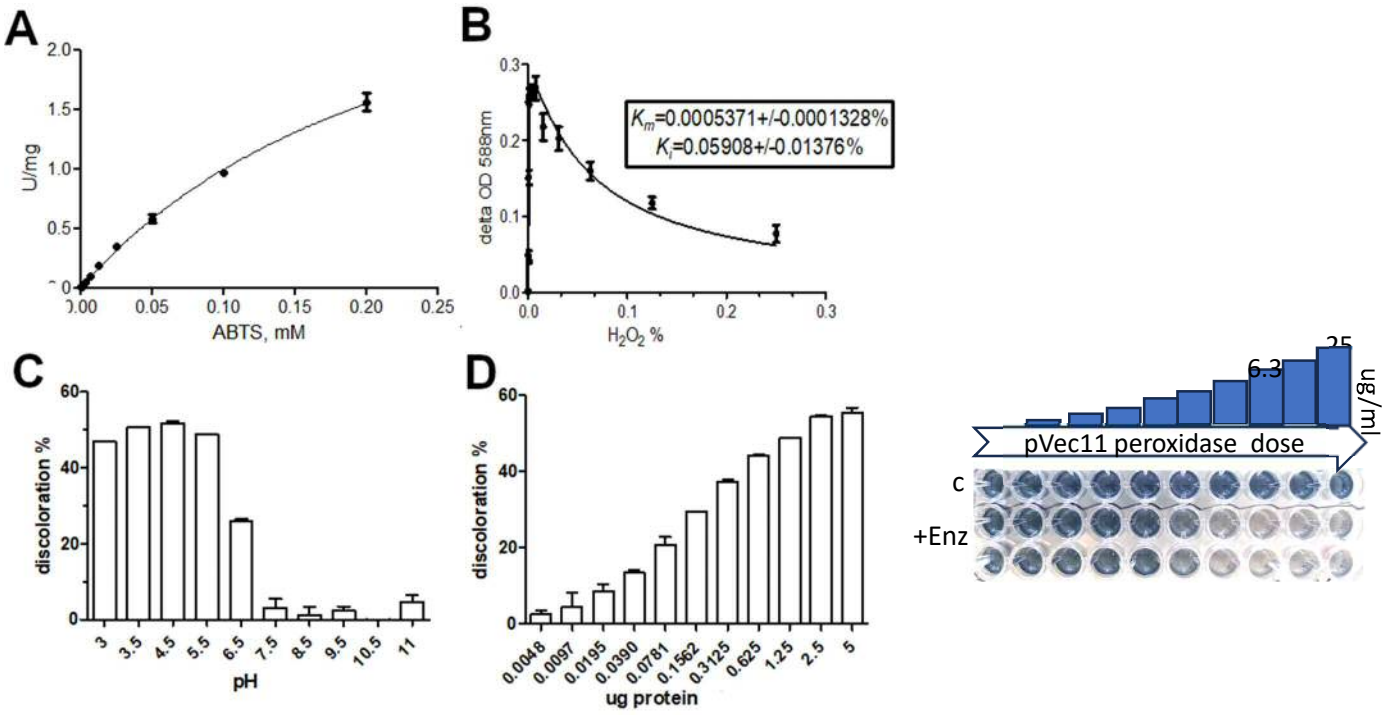
Application 2: Textiles (Dye removal)

Recommended application conditions (from research perspective):			
Temperature [°C]*:	[30C]	Substrate:	[dye from Shoeller, H_2O_2]
pH [-]*:	[3.5-5.5]	Substrate concentration [unit]: [description of the unit]	[5% dye, 0.004% H_2O_2,]
Medium (water, buffer, salt, cofactors, etc.)*:	[50mM buffer pH 4, 5, ,0.004% H_2O_2]	Expected product(s) and their quantities:	[unknown] under conditions described up to 55% of dye decolorates
Time / duration:	[2h]	Analytical methods:	[spectrophotometric absorbance at 588nm]
Enzyme concentration [ug/ml]: [description of the unit]	[25ug/ml]	Other remarks on the set-up of the enzyme reaction:	[text]
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

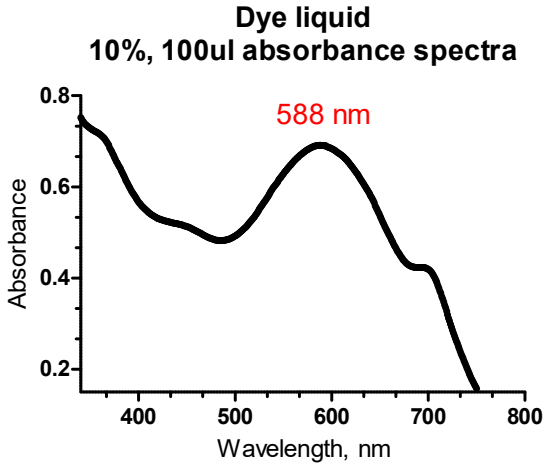
Brief description of observed activity (under recommended conditions):	[text]
Detailed methodology of activity assay:	• [Prepare the reaction mixture in 96-well microplates adding 10 µl of concentrated dye, 0.008% H2O2 (final concentration,freshly diluted) and water to 200ul taking into account the enzyme volume (5-10ug) in 50mM buffer (I used Britton Robinson buffer) pH 4.5, 30C • Control wells does not contain enzyme, but the elution buffer. • Mix by pipetting / plate shaking • Add 5-10ug of purified protein to fit in the final volume 200ul • Mix by pipetting and read the 0 time point at 588 nm, save the data • Seal plate with adhesive seal and keep shaking for 2h or overnight at 30C, 500rpm (or run the kinetic read every minute with 10 sec shaking between reads for 2 h without seal). • Read at 588nm • Subtract the control.
Verified positive and negative controls for the reaction:	[text]

Tested range of conditions:			
Temperature [°C]:	[30C]	Substrate:	[text]
pH [-]:	[3-11]	Substrate concentration [unit]:	[text]
Medium (water, buffer, salt, cofactors, etc.):	[H_2O_2 0.255-0.0002% tested K_i=0.0059%]	Expected product(s) and their quantities:	[text]
Time / duration:	[30min-2h]	Analytical methods:	[text]
Enzyme concentration [ug/ml]:	[25]	Other remarks:	[text]

Attachment (additional figures, descriptions, etc.)



Annex Fig. 17. pVec11 peroxidase characterisation using spectrophotometric assay at 405nm measured for ABTS, 588nm for 5%dye. Reaction volume 200ul, Britton-Robinson buffer 50mM, 30-120 min read, 30C. Effect of:(A) ABTS concentration, (B) H_2O_2 concentration in presence of 5% dye, (C) pH on pVec11 activity. (D) protein dosage effect on the dye discoloration



Annex Fig. 18. Shoeller dye absorbance spectra

pVec11 E.coli BL21DE3 Lobstr expression protocol:

- ½ TB media supplemented with 4% glycerol. Aerobic growth at 200rpm, 37C, ampicillin 100mg/L until OD600nm reach 0.6-0.8. 0.4mM IPTG for induction. Heme 10 mg (dissolved by NaOH Titration)/L of culture is added at induction step. After induction: 37C 3h 200rpm, followed by 16C, ON at 200rpm.
- Protein purification: Ni-NTA resin. (Our construct contains N-terminal His tag)

Enzyme specification sheet

Original version:	16.01.2024	UDUS	Rebecka Molitor	r.molitor@fz-juelich.de
Last update:	18.01.2024	UDUS	Rebecka Molitor	r.molitor@fz-juelich.de

Enzyme:	**#06**	**EstLip_TBEc304 (Biosynth preparation)**
Enzyme class:	List of enyzme preparations (quantities for industrial tests have already been shared with partners):	Target application sector(s):
Esterase / Lipase	2. 03917723SS1006| Biosynth | ESTLip_TBEc304	☒ Detergents ☒ Textiles (specify) ☒ Oil removal ☐ Dye removal ☐ Cosmetics ☐ Other:_____

Enzyme origin

Identifying partner:	UDUS	Type of expression:	Intracellular	Mode of application:	Solubilised enzyme
Original host:	*E. coli*	Purification and formulation:	Other: Purified from cells, stored in buffer	Measured activity [unit]:	[text]
Further characteristics / comments:	[text]				

Production batch: 03917723SS1006

Producing partner:	Biosynth	Type of expression:	Secreted	Mode of application:	Soluble enzyme
Production host:	*Pichia pastoris*	Purification and formulation:	Lyophylised whole cells	Measured activity:	[text]
Further characteristics / comments:	[text]				

Production batch: **[Batch number]**

Producing partner:	[institution]	Type of expression:	Choose	Mode of application:	Choose
Production host:	[host organism]	Purification and formulation:	[formulation] [enyzme fraction]	Measured activity:	[text]
Further characteristics / comments:	[text]				

Application 1: Detergents

Recommended application conditions (from research perspective):			
Temperature [°C]*:	40°C	Substrate:	Beef fat and lipstick
pH [-]*:	7-8	Substrate concentration [g/L]: [g_{fabric}/L_{buffer}]	80 g/L
Medium (water, buffer, salt, cofactors, etc.)*:	0.1 M Phosphate buffer, Surfactant: 1% Triton -X	Expected product(s) and their quantities:	Expected products: Fatty acids Measured products: free long chain fatty acids
Time / duration:	24h	Analytical methods:	NEFA free long chain fatty acid detection Kit,
Enzyme concentration [mg]: [$mg_{lyophilised\ material\ per\ reaction}$]	0.02-0.05 mg	Other remarks on the set-up of the enzyme reaction:	- Not stable in Henkel washing liquor
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

Brief description of observed activity (under recommended conditions):	- Kpi and HEPES buffer showed with and without enzyme activity towards the substrate beef fat (Annex Fig. 20) → might be false positive and will be repeated. - With the substrate lipstick the Kpi buffer negative control showed little activity after 24 h (Annex Fig. 21) - All enzyme concentrations show after 24 h as much activity as the positive control (Annex Fig. 21)
Detailed methodology of activity assay:	- Given amounts of enzymes refer to mg material per reaction. - Substrates beef fat and lipstick - Triton-X as supplement to solubilize long chain fatty acids. - Method: Incubation of enzyme + 100 µL buffer + fabric in MTP-wells at 40°C + free long chain fatty acid quantification in the reaction solution using NEFA-KIT - To check for buffer effects, we determined fatty acid release also with CSIC ‘s buffer.
Verified positive and negative controls for the reaction:	- Negative control: fabric in buffer+ Triton-X only, fabric in buffer with detergent without enzymes - Positive control: oleic acid in buffer, fabric with detergent with enzymes

Tested range of conditions:			
Temperature [°C]:	[text]	Substrate:	Beef fat and lipstick standard pollution
pH [-]:	7-8	Substrate concentration [g/L]:	ca. 8 g/L
Medium (water, buffer, salt, cofactors, etc.):	0.1 M Phosphate buffer 40 mM HEPES buffer+150mM salt, if applicable: Surfactant: 1% Triton -X	Expected product(s) and their quantities:	Fatty acids, dicarboxylic acids
Time / duration:	0.5h-24h	Analytical methods:	NEFA free long chain fatty acid detection Kit, acidification,
Enzyme concentration [mg]:	0.05-0.02 mg	Other remarks:	[text]

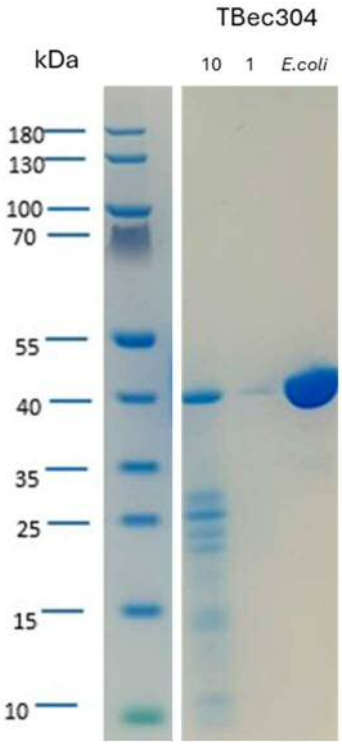
Application 2: Textiles (Oil removal)

Recommended application conditions (from research perspective):			
Temperature [°C]*:	40°C	Substrate:	61488Z ROH (barely any release of fatty acids from the other fabric samples)
pH [-]*:	7.2	Substrate [g/L] [g_{fabric}/L_{buffer}]	80 g/L
Medium (water, buffer, salt, cofactors, etc.)*:	0.1 M Phosphate buffer, Surfactant: 1% Triton -X	Expected product(s) and their quantities:	Expected products: Fatty acids Measured products: free long chain fatty acids
Time / duration:	24 h	Analytical methods:	NEFA free long chain fatty acid detection Kit,
Enzyme concentration [mg]: [mg enzyme per reaction]	0.09-0.18 mg	Other remarks on the set-up of the enzyme reaction:	Activity expected towards: Triglycerides, wax esters, esters in general, Polyester
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

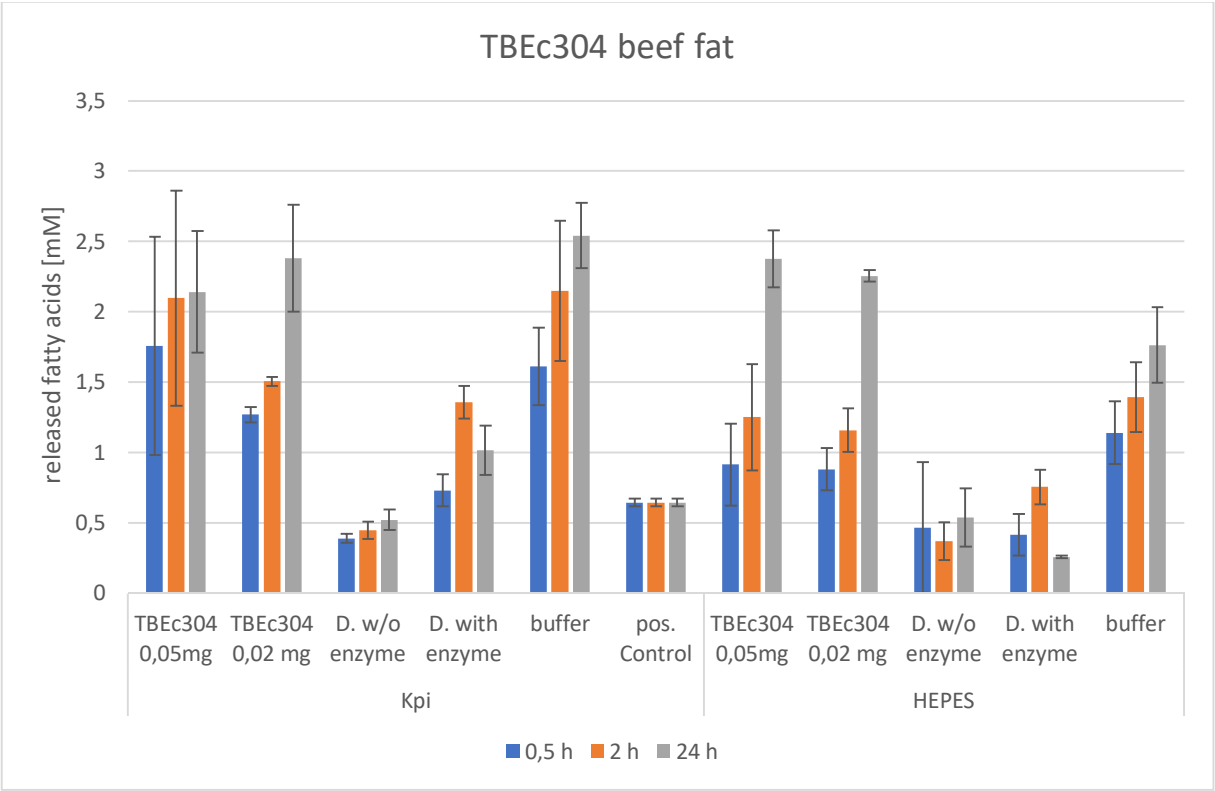
Brief description of observed activity (under recommended conditions):	0.09-0.18 mg of enzyme solution showed minor activity after 24 h (Annex Fig. 22) Overall activity after 24 h was higher in Kpi buffer but in HEPEs buffer activity was already there after 0.5 h (Annex Fig. 22)
Detailed methodology of activity assay:	- Given amounts of enzymes refer to mg material per reaction - Schoeller-fabric Nr. 61488Z ROH was chosen because it leads to the clearest signals in previous experiments - Triton-X as supplement to solubilize long chain fatty acids - Method: Incubation of enzyme + 100 µL buffer + fabric in MTP-wells at 40°C + free long chain fatty acid quantification in the reaction solution using NEFA-KIT - To check for buffer effects, we determined fatty acid release also with CSIC 's buffer (HEPES).
Verified positive and negative controls for the reaction:	- Positive control: oleic acid in buffer - Negative control: buffer without enzyme

Tested range of conditions:			
Temperature [°C]:	40°C	Substrate:	61488Z ROH (barely any release of fatty acids from the other fabric samples)
pH [-]:	7-8	Substrate concentration [g/L]:	80 g/L
Medium (water, buffer, salt, cofactors, etc.):	0.1 M Phosphate buffer 40 mM HEPES buffer+150mM salt, if applicable: Surfactant: 1% Triton -X	Expected product(s) and their quantities:	Fatty acids, dicarboxylic acids
Time / duration:	0.5 h-24 h	Analytical methods:	NEFA free long chain fatty acid detection Kit, acidification,
Enzyme concentration [mg]:	0.0288-0.18 mg	Other remarks:	[text]

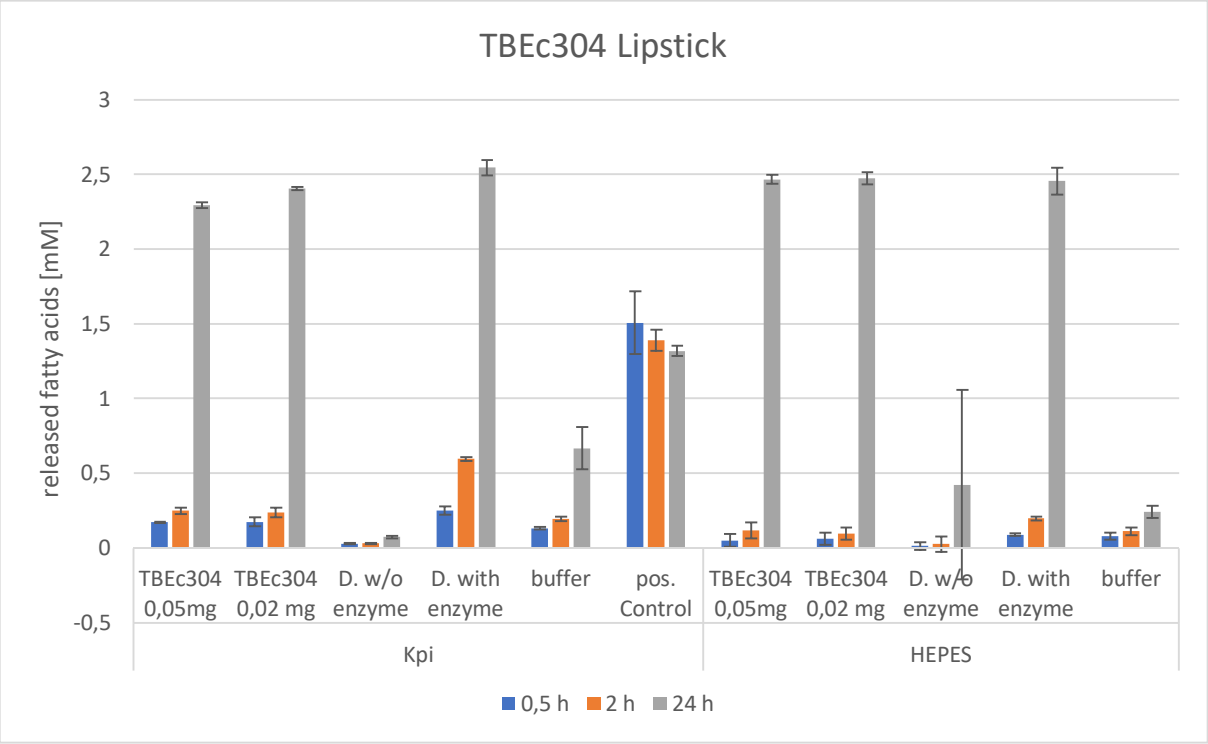
Attachment (additional figures, descriptions, etc.)



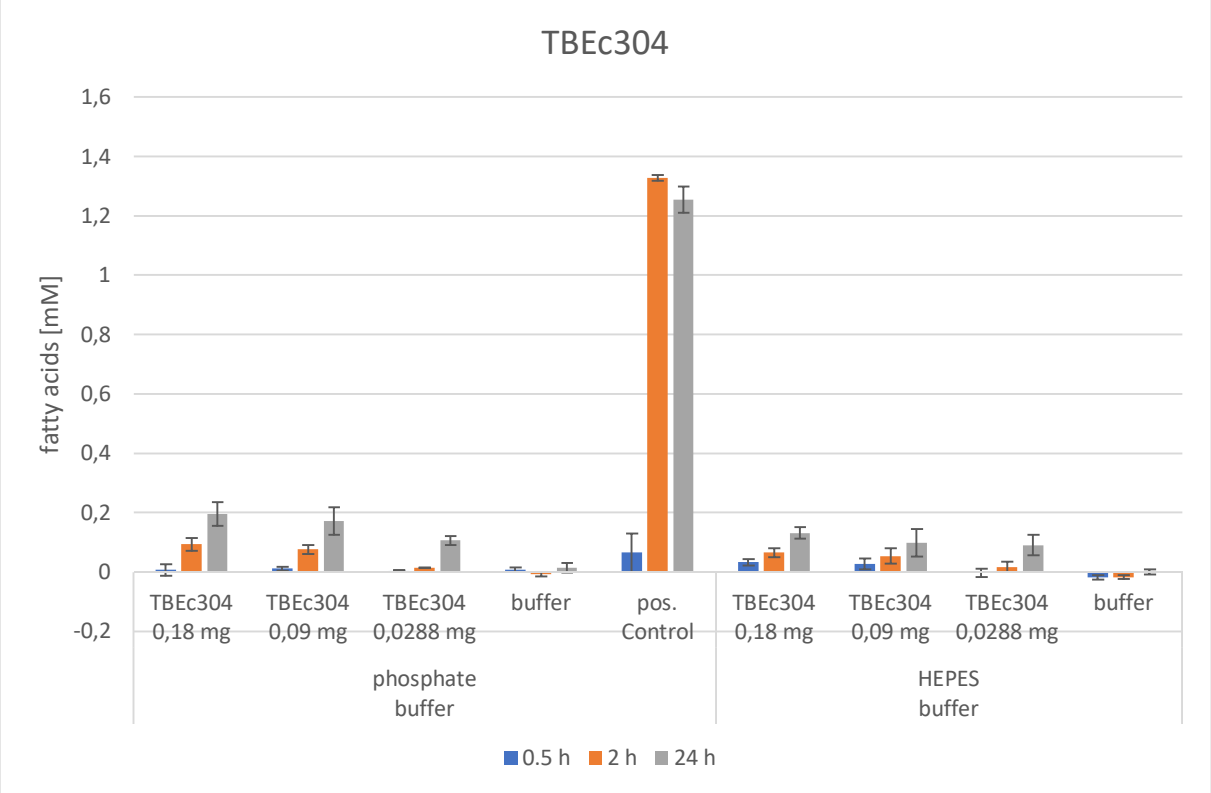
Annex Fig. 19. SDS-PAGE of Pichia pastoris and E.coli enzymes, concentrations of 10 mg/ml and 1 mg/ml (Pichia), E. coli produced enzyme undiluted as reference. 5 µl applied.



Annex Fig. 20. TBec304tested with beef fat standard pollution incubated in HEPES and Kpi buffer between 0.5 and 24 h. The enzyme was tested in two different concentrations and with the addition of detergent (D.). As negative controls the detergent was tested without enzymes as well as only the buffer.



Annex Fig. 21. TBec304tested with lipstick standard pollution incubated in HEPES and Kpi buffer between 0.5 and 24 h. The enzyme was tested in two different concentrations and with the addition of detergent (D.). As negative controls the detergent was tested without enzymes as well as only the buffer.



Annex Fig. 22. TBec304 tested with the substrate textile 61488Z ROH for 0.5-24 h at 40°C in Kpi buffer and HEPES buffer.

Additional information is shown in Supplementary Material (Supplementary Fig. 4).

Enzyme specification sheet

Original version:	03.11.2023	UDUS	Stephan Thies Rebecka Molitor	s.thies@fz-juelich.de r.molitor@fz-juelich.de
Last update:	[date]	[institution]	[Name]	[email]

Enzyme:	**#07**	**Paes_PE-H Y250S**
Enzyme class:	List of enzyme preparations (quantities for industrial tests have already been shared with partners):	Target application sector(s):
Esterase / Lipase / PETase	3. Batch 03908423SC0609 | Biosynth] | Paes-PE-H-Y250S]	☒ Detergents ☒ Textiles (specify) ☒ Oil removal ☐ Dye removal ☒ Polyester end of life ☐ Cosmetics ☐ Other:_____

Enzyme origin

Identifying partner:	UDUS	Type of expression:	Intracellular	Mode of application:	Solubilised enzyme
Original host:	*E.coli*	Purification and formulation:	Other (specify) Purified from cells, stored in buffer	Measured activity [unit]:	ImpranilDLN Polyester https://www.frontiersin.org/article/10.3389/fmicb.2020.00114/full
Further characteristics / comments:	- *E*. coli-derived protein losts activity in high concentration (highest specific activity at <100nM = 3mg/L) - C-term His-Tag for purification				

Production batch: 03908423SC0609

Producing partner:	Biosynth	Type of expression:	Secreted	Mode of application:	Soluble enzyme
Production host:	*Pichia pastoris*	Purification and formulation:	Lyophylised Supernatant	Measured activity:	pNP butyrate
Further characteristics / comments:	Material shows good enzyme content (Annex Fig. 23)				

Production batch: [Batch number]

Producing partner:	[institution]	Type of expression:	Choose	Mode of application:	Choose
Production host:	[host organism]	Purification and formulation:	[formulation] [enyzme fraction]	Measured activity:	[text]
Further characteristics / comments:	[text]				

Application 1: Textiles (Oil removal)

Recommended application conditions (from research perspective):			
Temperature [°C]*:	30°C or 40°C	Substrate:	61488Z ROH
pH [-]*:	7-8	Substrate concentration [unit]: [description of the unit]	Ca. 8 mg fabric/100µl reaction = 80 g fabric/L
Medium (water, buffer, salt, cofactors, etc.)*:	0.1 M Phosphate buffer or HEPES buffer, 100-150mM salt, Surfactant: 1% Triton - X	Expected product(s) and their quantities:	Expected products: fatty acids/carboxylic acids. Measured products: free long chain fatty acids
Time / duration:	2h	Analytical methods:	NEFA free long chain fatty acid detection Kit,
Enzyme concentration [unit]: [description of the unit]	0.3-15 g/L lyophilised material Good results already with 0.9 g/L (Annex Fig. 24) =ca. 11,25 mg powder/g fabric	Other remarks on the set-up of the enzyme reaction:	- Activity expected towards: Triglycerides, polyesters - Low but measurable activity towards PET
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

Brief description of observed activity (under recommended conditions):	See Annex Fig. 24
Detailed methodology of activity assay:	- Given amounts of enzymes refer to mg material per reaction - Schoeller-fabric Nr. 61488Z ROH was chosen because it leads to the clearest signals in previous experiments) - Triton-X as supplement to solubilize long chain fatty acids - Method: Incubation of enzyme + 100 µL buffer + fabric in MTP-wells at 30°C + free long chain fatty acid quantification in the reaction solution using NEFA-KIT Also: check for fatty acid release after 2h of incubation also with CSIC' buffer.
Verified positive and negative controls for the reaction:	- [Negative control: fabric in Buffer+ Triton-X only. - positive control: Dim008

Tested range of conditions:			
Temperature [°C]:	Melting Point:55°C	Substrate:	Tributyrin, Olive oil, coconut oil , dibutyl sebacate, Fabric sample2 ROH, (barely any release of fatty acids from the other fabric samples)
pH [-]:	[text]	Substrate concentration [unit]:	1-10mM (= up to ca. 8g/L) 80g/L for fabrics
Medium (water, buffer, salt, cofactors, etc.):	0.1 M Phosphate buffer 40 mM HEPES buffer+ 150mM salt,	Expected product(s) and their quantities:	Fatty acids, dicarboxylic acids

	if applicable: Surfactant: 1% Triton - X		
Time / duration:	10min-24h	Analytical methods:	NEFA free long chain fatty acid detection Kit, acidification,
Enzyme concentration [unit]:	0.0015-15g/L	Other remarks:	[text]

Application 2: Detergents

Recommended application conditions (from research perspective):			
Temperature [°C]*:	30°C or 40°C	Substrate:	PC-S-16 Lipstick, pink, Material: Polyester/Cotton (PCN-01), PC-S-61B Beef fat colored with violet dye, Material: Polyester/Cotton (PCN-01),
pH [-]*:	7-8	Substrate concentration [unit]: [description of the unit]	8 mg fabric/reaction vessel = 80 g fabric/L
Medium (water, buffer, salt, cofactors, etc.)*:	0.1 M Phosphate buffer or HEPES buffer, 100-150mM salt, Surfactant: 1% Triton - X	Expected product(s) and their quantities:	Expected products: fatty acids/carboxylic acids. Measured products: free long chain fatty acids
Time / duration:	24h	Analytical methods:	NEFA free long chain fatty acid detection Kit,
Enzyme concentration [unit]: [description of the unit]	0.3-1.8 g/L lyophilised material Good results already with 0.9 g/L (Annex Fig. 24) =ca. 11.25 mg powder/ g fabric	Other remarks on the set-up of the enzyme reaction:	- Moderately stable in Henkel washing liquor - Activity expected towards: Triglycerides, polyesters - Low but measurable activity towards PET
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

Brief description of observed activity (under recommended conditions):	See Annex Fig. 25
Detailed methodology of activity assay:	- Given amounts of enzymes refer to mg material per reaction - Triton-X as supplement to solubilize long chain fatty acids Method: Incubation of enzyme + 100 µL buffer + fabric in MTP-wells at 30°C + free long chain fatty acid quantification in the reaction solution using NEFA-KIT
Verified positive and negative controls for the reaction:	- Negative control: fabric in Buffer+ Triton-X only. - positive control: Henkel washing liquor+enzymes

Tested range of conditions:			
Temperature [°C]:	Melting Point:55°C	Substrate:	Tributyrin, Olive oil, coconut oil , dibutyl sebacate
pH [-]:	[text]	Substrate concentration [unit]:	1-10mM (= up to ca. 8g/L) 80g/L for fabrics
Medium (water, buffer, salt, cofactors, etc.):	0.1 M Phosphate buffer 40 mM HEPES buffer+ 150mM salt, if applicable: Surfactant: 1% Triton - X	Expected product(s) and their quantities:	Fatty acids, dicarboxylic acids

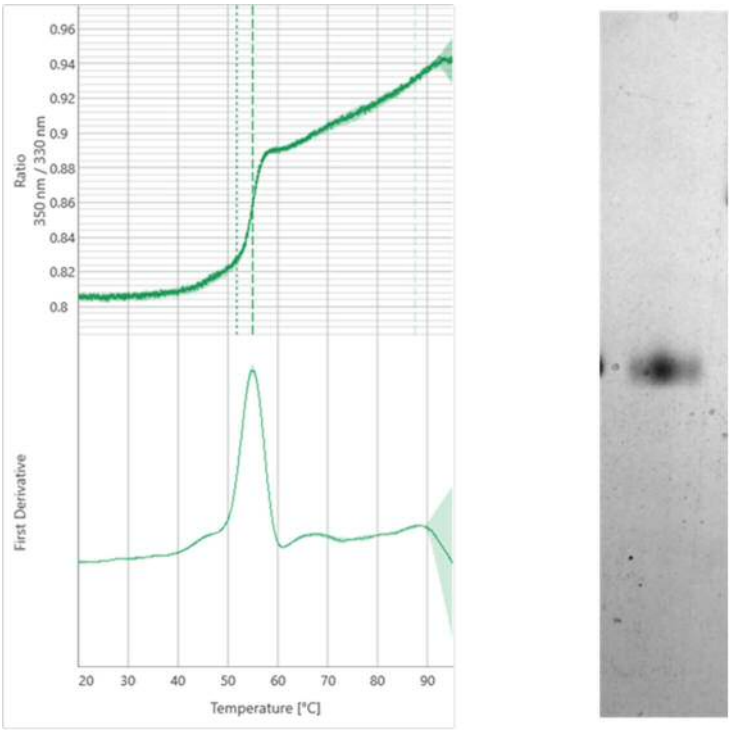
Time / duration:	10min-24h	Analytical methods:	NEFA free long chain fatty acid detection Kit, acidification,
Enzyme concentration [unit]:	0.0015-	Other remarks:	[text]

Application 3: Textiles (polyester end of life/modification)			
Recommended application conditions (from research perspective):			
Temperature [°C]*:	30°C or 40°C	Substrate:	3X58 (VORB)
pH [-]*:	7-8	Substrate concentration [unit]: [description of the unit]	40 mg/L
Medium (water, buffer, salt, cofactors, etc.)*:	0.1 M Phosphate buffer. 5% DMSO	Expected product(s) and their quantities:	Mono-and oligomers of polyester
Time / duration:	24h-48h	Analytical methods:	HPLC-UV/Vis for monomers
Enzyme concentration [unit]: [description of the unit]	0.3 g/L	Other remarks on the set-up of the enzyme reaction:	Activity to PET fabric is still rather low
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

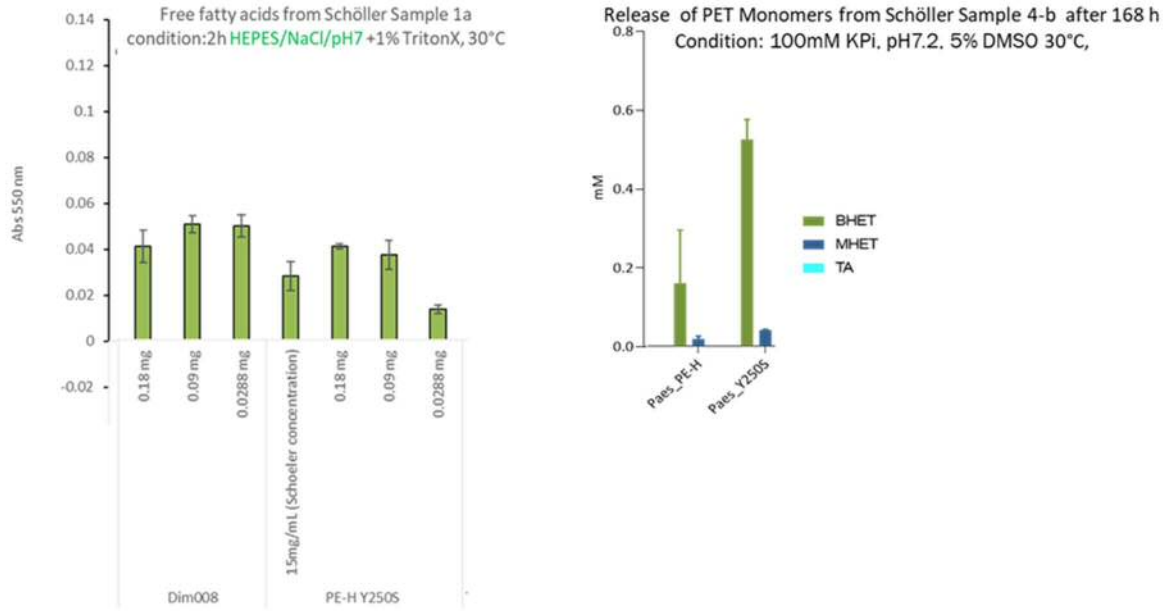
Brief description of observed activity (under recommended conditions):	See Annex Fig. 24, right side
Detailed methodology of activity assay:	(10.3389/fmicb.2020.00114)
Verified positive and negative controls for the reaction:	Negative: Material in 0.1 M Phosphate buffer. 5% DMSO

Tested range of conditions:			
Temperature [°C]:	Melting Point:55°C	Substrate:	Aliphatic Polyester-polyurethan foam and coating, PET plastic (10.3389/fmicb.2020.00114)
pH [-]:	[text]	Substrate concentration [unit]:	[text]
Medium (water, buffer, salt, cofactors, etc.):	0.1 M Phosphate buffer, 5% -10% DMSO,100mM NaCl	Expected product(s) and their quantities:	Mono-and oligomers of polyesters
Time / duration:	10min-24h	Analytical methods:	Dependent on applied polymer: Acidification, turbidity clearing, HPLC-UV/Vis
Enzyme concentration [unit]:	0.15-2.4-g/L	Other remarks:	[text]

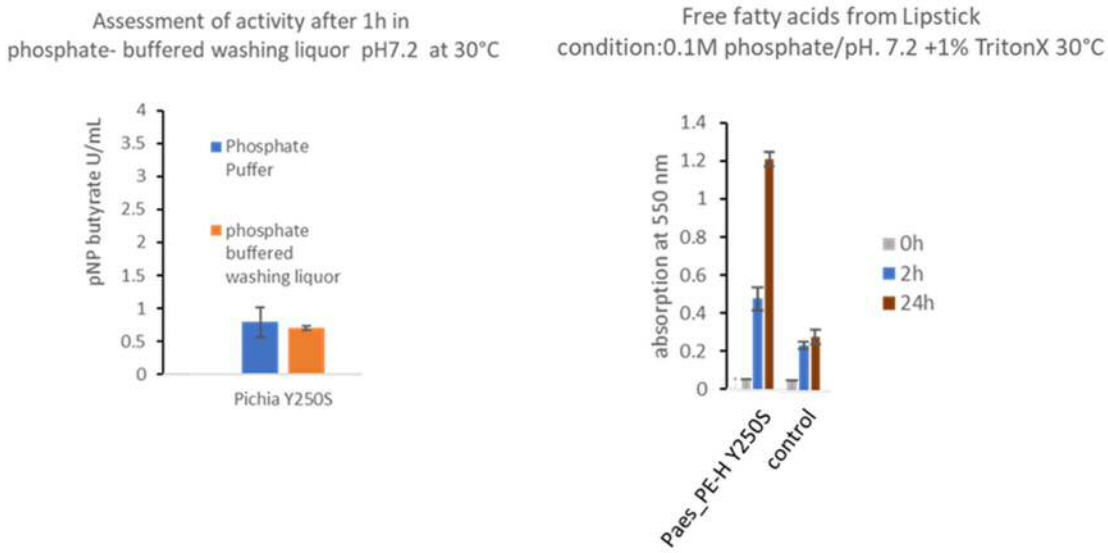
Attachment (additional figures, descriptions, etc.)



Annex Fig. 23. Left: Melting curves of Biosynth lyophilised material using Differential Scanning fluorometry, indicating a melting point at 55°C. Right: Coomassie stained SDS page of 1 mg/mL lyophilised material.



Annex Fig. 24. Left: Release of fatty acids from sample 1a (61488Z ROH) in CSIC Buffer (40 mM HEPES, pH,7, 150 mM salt) with Triton X as a surfactant to solubilize fatty acids. It confirms activity to the oils on the fabric. Stated enzyme amount represent the absolute amount of enzyme powder in 100µL reaction. Right: Concentration of released PET monomers from from Schöller Sample 4-b 3X58 (VORB, 100% PES 100g/m2) after 168 h. This experiment was done with the E. coli preparation in 100mM Potassium phosphate buffer, pH7.2, 5% DMSO, 30°C.



Annex Fig. 25. Paes_PE-H for detergent application. Left: Residual activity in washing liquor indicates engineering demand of this lipase. Right: Fatty acid release from Lipstick-soiled textiles indicates activity against fatty stains in the presence of the milder surfactant TritonX. This assay was done with the E. coli preparation.

Additional information is shown in Supplementary Material (Supplementary Fig. 3, 10).

Enzyme specification sheet

Original version:	03.11.2023	UDUS	Stephan Thies Rebecka Molitor	s.thies@fz-juelich.de r.molitor@fz-juelich.de
Last update:	[date]	[institution]	[Name]	[email]

Enzyme:	**#08**	**PtEst**

Enzyme class:	List of enzyme preparations (quantities for industrial tests have already been shared with partners):	Target application sector(s):
Esterase / Lipase	4. Batch 03911523SC0728 | Biosynth | EstLip-PtEst	☒ Detergents ☒ Textiles (specify) ☒ Oil removal ☐ Dye removal ☐ Cosmetics ☐ Other:_____

Enzyme origin

Identifying partner:	UDUS	Type of expression:	Intracellular	Mode of application:	Solubilised enzyme
Original host:	*E.coli*	Purification and formulation:	Other (specify) purified from cell extract Wählen Sie ein Element aus.	Measured activity [unit]:	pNP-butyrate
Further characteristics / comments:	C-term His-Tag for purification				

Production batch: 03911523SC0728

Producing partner:	Biosynth	Type of expression:	Secreted	Mode of application:	Soluble enzyme
Production host:	*Pichia pastoris*	Purification and formulation:	Lyophylised Supernatant	Measured activity:	pNP butyrate
Further characteristics / comments:	Material shows comparably low enzyme content (Annex Fig. 26)				

Production batch: [Batch number]

Producing partner:	[institution]	Type of expression:	Choose	Mode of application:	Choose
Production host:	[host organism]	Purification and formulation:	[formulation] [enyzme fraction]	Measured activity:	[text]
Further characteristics / comments:	[text]				

Application 1: Textiles (Oil removal)

Recommended application conditions (from research perspective):			
Temperature [°C]*:	30°C or 40°C	Substrate:	61488Z ROH
pH [-]*:	7-8	Substrate concentration [unit]: [description of the unit]	Ca. 8 mg fabric/100µl reaction = 80 g fabric/L
Medium (water, buffer, salt, cofactors, etc.)*:	0.1 M Phosphate buffer, Surfactant: 1% Triton -X	Expected product(s) and their quantities:	Expected products: mainly short und middle chain length fatty acids
Time / duration:	24 h	Analytical methods:	NEFA free long chain fatty acid detection Kit,
Enzyme concentration [unit]: [description of the unit]	0.3-1.8 g/L lyophilised material, Best results already with 0.3 g/L (Annex Fig. 27) =ca. 3.75mg powder/g fabric	Other remarks on the set-up of the enzyme reaction:	- not a lipase - Activity expected towards: Triglycerides with C4-10 , large variety of short chained and aromatic esters in general
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

Brief description of observed activity (under recommended conditions):	See Annex Fig. 27
Detailed methodology of activity assay:	- Given amounts of enzymes refer to mg material per reaction - Schoeller-fabric Nr. 61488Z ROH was chosen because it lead to the clearest signals in previous experiments) - Triton-X as supplement to solubilize long chain fatty acids - Method: Incubation of enzyme + 100 µL buffer + fabric in MTP-wells at 30°C + free long chain fatty acid quantification in the reaction solution using NEFA-KIT To check for buffer effects, we determined fatty acid release after 2h of incubation also with CSIC's buffer.
Verified positive and negative controls for the reaction:	- Negative control: fabric in Buffer+ Triton-X only. - positive control: Dim008,

Tested range of conditions:			
Temperature [°C]:	Melting Point:57°C (=max-temperature)	Substrate:	Tributyrin, dibutyl sebacate, Polycaprolactone (530Da), Diesters of furandicarboxyl acid and terephthalic acid Further substrate profile in 10.1111/febs.15680
pH [-]:	5-9 (10.1111/febs.15680)	Substrate concentration [unit]:	1-10mM (= up to ca. 8g/L)
Medium (water, buffer, salt, cofactors, etc.):	0.1 M Phosphate buffer 40mM HEPES buffer+ 150mM salt, if applicable: Surfactant: 1% Triton -X	Expected product(s) and their quantities:	Fatty acids, dicarboxylic acids

Time / duration:	0.5h-24h	Analytical methods:	NEFA free long chain fatty acid detection Kit, acidification
Enzyme concentration [unit]:	0.3-1.8 g/L 0.6g/L	Other remarks:	

Application 2: Detergents

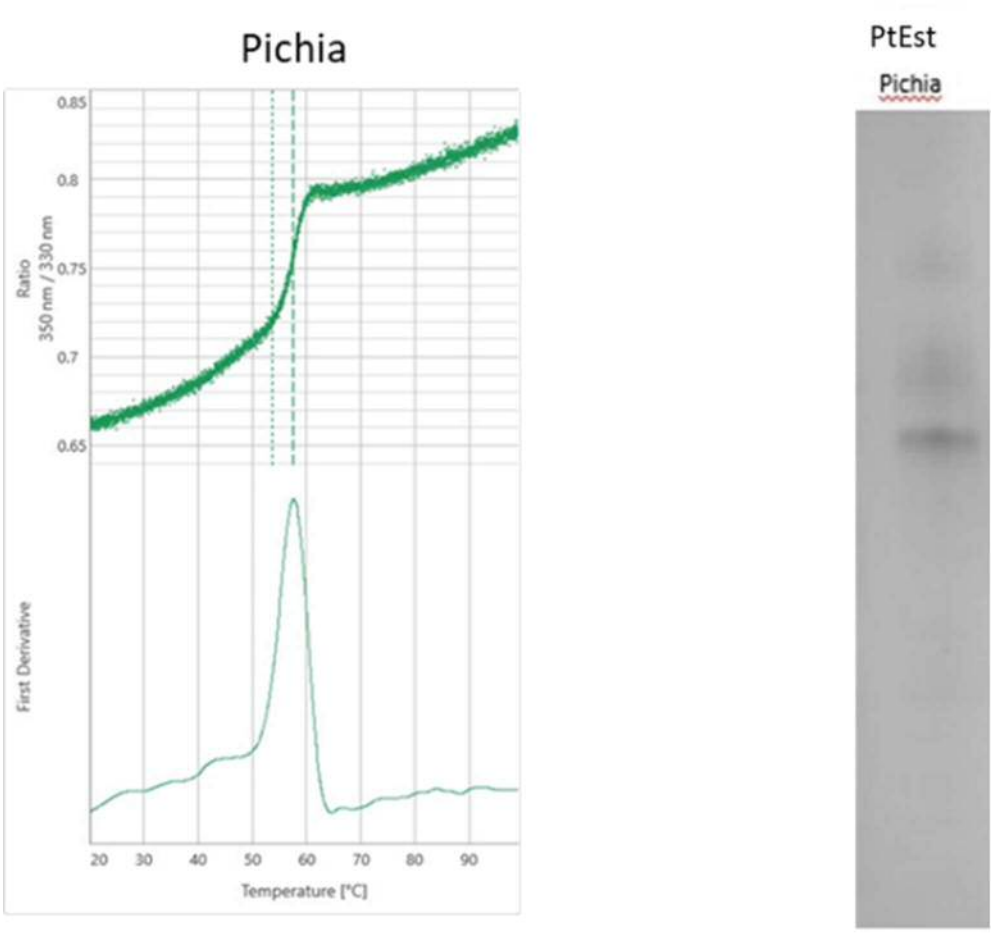
Recommended application conditions (from research perspective):			
Temperature [°C]*:	40°C	Substrate:	PC-S-16 Lipstick, pink, Material: Polyester/Cotton (PCN-01),
pH [-]*:	7-8	Substrate concentration [unit]: [description of the unit]	8 mg fabric/reaction vessel = 80 g fabric/L
Medium (water, buffer, salt, cofactors, etc.)*:	0.1 M Phosphate buffer, Surfactant: 1% Triton -X	Expected product(s) and their quantities:	Expected products: mainly short und middle chain length fatty acids
Time / duration:	24 h	Analytical methods:	NEFA free long chain fatty acid detection Kit,
Enzyme concentration [unit]: [description of the unit]	0.2 g/L & 0.5g/L lyophilised material	Other remarks on the set-up of the enzyme reaction:	- Moderately stable in Henkel washing liquor, - Only minor activity toward fatty strains
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

Brief description of observed activity (under recommended conditions):	See Annex Fig. 28
Detailed methodology of activity assay:	- Given amounts of enzymes refer to mg material per reaction - Triton-X as supplement to solubilize long chain fatty acids - To check for buffer effects, we determined fatty acid release after 2h of incubation also with CSIC' buffer. Method: Incubation of enzyme + 100 µL buffer + fabric in MTP-wells at 30°C + free long chain fatty acid quantification in the reaction solution using NEFA-KIT
Verified positive and negative controls for the reaction:	- Negative control: fabric in Buffer+ Triton-X only. positive control: Henkel washing liquor+enzymes

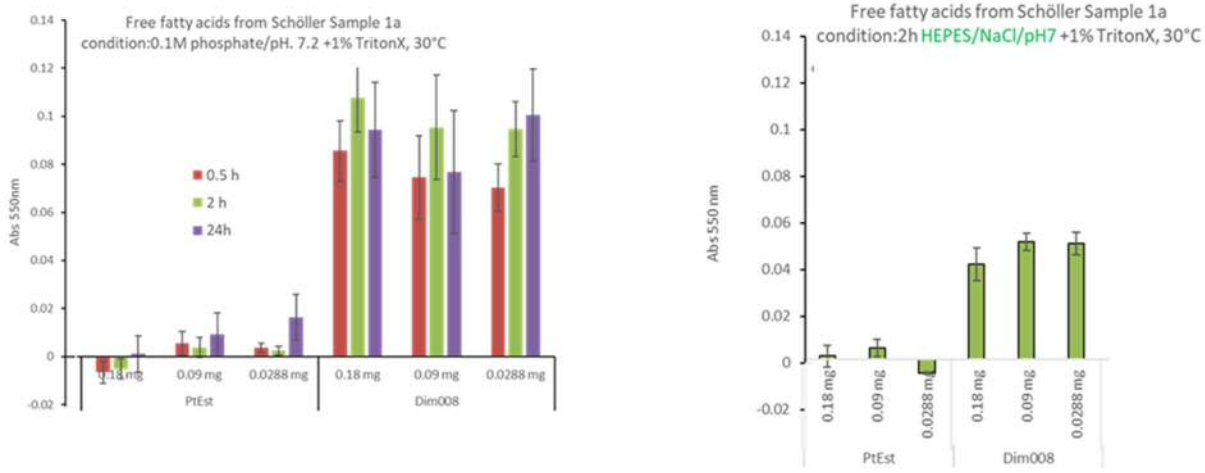
Tested range of conditions:			
Temperature [°C]:	Melting Point:57°C (=max-temperature)	Substrate:	[PC-S-16 Lipstick, pink, Material: Polyester/Cotton (PCN-01), PC-S-61B Beef fat colored with violet dye, Material: Polyester/Cotton (PCN-01), Tributyrin, dibutyl sebacate, Polycaprolactone (530Da), Diesters of furandicarboxyl acid and terephthalic acid Further substrate profile in 10.1111/febs.15680
pH [-]:	5-9 (10.1111/febs.15680)	Substrate concentration [unit]:	1-10mM (= up to ca. 8g/L)
Medium (water, buffer, salt, cofactors, etc.):	0.1 M Phosphate buffer 40mM HEPES buffer+ 150mM salt,	Expected product(s) and their quantities:	Fatty acids, dicarboxylic acids

	if applicable: Surfactant: 1% Triton - X		
Time / duration:	0.5h-24h	Analytical methods:	NEFA free long chain fatty acid detection Kit, acidification
Enzyme concentration [unit]:	0.2-0.5 g/L lyophilised material	Other remarks:	

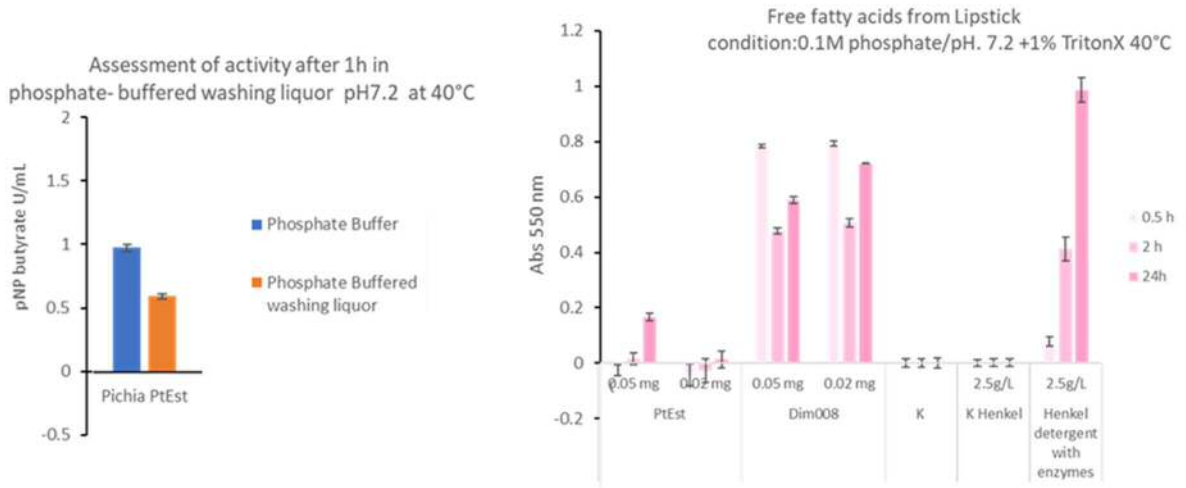
Attachment (additional figures, descriptions, etc.)



Annex Fig. 26. Left: Melting curves of Biosynth lyophilised material using Differential Scanning fluorometry, indicating a melting point at 57°C. Right: Coomassie stained SDS page of **10mg/mL** lyophilised material indicating a rather low enzyme content of this preparation.



Annex Fig. 27. Release of fatty acids from sample 1a (61488Z ROH) in two different conditions (Output: signal intensity at 550nm). Left: 0.1 Potassium phosphate, pH7.2, no salt; right: CSIC Buffer (40 mM HEPES, pH,7, 150 mM salt), each with Triton X as a surfactant to solubilize fatty acids. It confirms only minor activity to the oils on the fabric and further shows decreased activity of this enzyme under the HEPES/pH7/salt conditions. Stated enzyme amounts represent the absolute amount of enzyme powder in 100µL reaction.



Annex Fig. 28. PtEst for detergent application. Left: Residual activity in washing liquor indicates engineering demand of this esterase. Right: Fatty acid release from Lipstick-soiled textiles after 0.5h, 2h, 24h indicates activity against fatty stains in the presence of the mild surfactant TritonX. Stated enzyme amount represent the absolute amount of enzyme powder in 100µL reaction.

Enzyme specification sheet

Original version:	23.10.2023	CSIC	Paula Vidal	p.vidal.ramon@csic.es
Last update:	28.10.2023	CSIC	Paula Vidal	p.vidal.ramon@csic.es

Enzyme:	**#09**	**FE_Polur1**

Enzyme class:	List of enzyme preparations (quantities for industrial tests have already been shared with partners):	Target application sector(s):
Esterase / Lipase / PETase	1. B01 | BIOSYNTH | Pp_ExCel (Lot: 03911323SC0726)	☒ Detergents ☒ Textiles (specify) ☒ Oil removal ☐ Dye removal ☐ Cosmetics ☐ Other:_____

Production batch: B01

Producing partner:	Biosynth	Type of expression:	Secreted	Mode of application:	Soluble enzyme
Production host:	*P. pastoris*	Purification and formulation:	Lyophylised Supernatant	Measured activity:	Glyceryl tributurate: 6.42 U/mg
Further characteristics / comments:	Activity measured for tributyrin (40°C, pH 8.0)				

Application 1: Detergents

Recommended application conditions (from research perspective):			
Temperature [°C]*:	40 °C (optimal) 30-45°C (acceptable) (Annex Fig. 29A)	Substrate:	Stained clothes PC-09, C-S-05S, P-S-16, C-S-17, PC-S-132, C-S-61, C-S-10
pH [-]*:	7.0-8.0 (optimal) 7.0-9.0 (acceptable) (Annex Fig. 29B)	Substrate concentration [g_C/L]: Grams of stained clothes per L of wash liquor	40 g_C/L
Medium (water, buffer, salt, cofactors, etc.)*:	Washing liquor (HENKEL®) (2.5 g/L)	Expected product(s) and their quantities: Grams of enzyme per L of wash liquor	Fat-free clothes (solid), glycerol (soluble), free fatty acids (soluble); exact composition unknown
Time / duration:	30 min	Analytical methods:	Free fatty acids: Colorimetric method using the NEFA-Kit.
Enzyme concentration [g_E/L]: Grams of enzyme per L of wash liquor	B01: 20 g_E/L	Other remarks on the set-up of the enzyme reaction:	Discuss with Henkel if measurement of the "cleanliness" of the clothes is required
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

Brief description of observed activity (under recommended conditions):	Polur1 lipase (B01) can be used for detergent applications, showing in washing liquour higher activity for some stained clothes compared to HENKEL® Liquid Laundry Detergent_A with enzymes (Annex Fig. 29C). Polur1 was also stable in the presence of washing liquour (Annex Fig. 30). It is also effective for stained clothes cleaning in buffer (Tris-HCl buffer at 40 mM), preferably at 30 °C (Annex Fig. 31) and for mayo stains (Annex Fig. 32).
Detailed methodology of activity assay:	Small-scale assays and analytics set-up by CSIC as follows: 1) Using a circle hole puncher, a small piece (5 mm diameter, 4 mg) of each of the stained clothes are cut [stained clothes include 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); 2) The small piece is added to a 2-ml safe-lock Eppendorf® polypropylene tubes (ref. 0030 120.094, Greiner Bio-One GmbH, Kremsmünster, Austria); 3) Then, 100 µl of washing liquor (2.5 g/L) are added; and 4) The appropriated amount of enzyme is added, and the reaction is maintained under the set-up experimental conditions, after which the release of fatty acids is determined by a colorimetric method using the NEFA-Kit (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany), as follows: in a 96 well plate, 10 µl of the reaction solution + 100 µl of NEFA solution 1 (R1a) are transferred. Following 6 min incubation at 37°C, 50 µl of NEFA solution 2 (R2a) are added. After 6 min incubation at 30°C, the samples' absorbance is measured at 550nm using a Synergy HT Multi Mode Microplate Reader (Agilent, Madrid, Spain). Assay Conditions at IST-ID: 30 ºC/40 ºC; pH 8; Tris-HCl buffer at 40 mM; Stains 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), cut into disks with 6 mm in diameter; Enzyme concentration, in reaction media, of

	0.075 g L-1. Testing Procedure: Disks, with a diameter of 6 mm, of standard stains were first cut with a hole puncher and weighed. Each disk placed in a 1.5 mL microtubes with 200 µL of Tris-HCl buffer at 40 mM, pH 8. 10 µL of enzyme solution at 1.5 mg mL-1 (prepared with Tris-HCl buffer at 40 mM) were then added to each tube; no enzymes were added to two replicas of each stain, as a control. Tubes were incubated for 24 h, at either 30 ºC or 40 ºC, in an orbital shaker at 200 rpm. After incubation, fatty acid concentration in the reaction media were evaluated with NEFA-HR(2) kit, according to standard procedure
Verified positive and negative controls for the reaction:	Positive control: HENKEL® Liquid Laundry Detergent_A including enzymes. Negative control: Washing liquor buffer (without enzymes)

Tested range of conditions:			
Temperature [°C]:	5-70°C (Annex Fig. 29A,B)	Substrate:	Stained clothes PC-09, C-S-05S, P-S-16, C-S-17, PC-S-132, C-S-61, C-S-10.
pH [-]:	4.0 - 11.0 (Annex Fig. 29C)	Substrate concentration [g_C/L]:	40 g_C/L
Medium (water, buffer, salt, cofactors, etc.):	Washing liquor (HENKEL®) (2.5 g/L)	Expected product(s) and their quantities:	Fat-free clothes (solid), glycerol (soluble), free fatty acids (soluble); exact composition unknown
Time / duration:	0.5-4 h	Analytical methods:	Free fatty acids: Colorimetric method using the NEFA-Kit. No other method tested.
Enzyme concentration [g_E/L]:	10-30 g_E/L	Other remarks:	Polur1 lipase (B01) is also effective for stained clothes cleaning in buffer (Tris-HCl buffer at 40 mM), at 30 °C and pH 8.0, at concentrations as low as 0.075 g L^{-1}.

Application 2: Textiles (Oil removal)

Recommended application conditions (from research perspective):			
Temperature [°C]*:	30-40 °C (optimal) 40-45°C (acceptable) (Annex Fig. 29A)	Substrate:	Schoeller fabrics 3X58, E031303, 61988F1, 5237-00, 61488F1, 67007 (all ROH fabrics). Not pretreated (raw).
pH [-]*:	7.0-8.0 (optimal) 7.0-9.0 (acceptable) (Annex Fig. 29B)	Substrate concentration [g_T/L]: (Grams of oil-containing textile per L of buffer solution)	34 g_T/L
Medium (water, buffer, salt, cofactors, etc.)*:	10-40 mM HEPES buffer (pH 7.0), or 10-40 mM K_2HPO_4 buffer (pH 7.0), or 40 mM Tris-HCl buffer (pH 8)	Expected product(s) and their quantities:	Oil-free textile (solid), glycerol (soluble), free fatty acids (soluble); exact composition unknown
Time / duration:	24 h – 65 h	Analytical methods:	Free fatty acids: Colorimetric method using the NEFA-Kit (indirect method) or GC (direct method).
Enzyme concentration [g_E/g_T]: (Grams of enzyme per gram of textile)	B01: 0.015 - 0.6 g_E/g_T	Other remarks on the set-up of the enzyme reaction:	

** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*

Brief description of observed activity (under recommended conditions):	We used as buffers 40 mM HEPES buffer pH 7.0 (by CSIC) or 40 mM Tris-HCl buffer pH 8.0 (by IST-ID), but we think any other buffer with pH 7.0-8.0 can be used. Polur1 lipase (B01) can be used for spinning oil removal in Schoeller fabrics, although it has low activity towards the tested fabrics when removal was determined indirectly by measuring the release of fatty acids to the reaction media as detected with the NEFA-kit. However, when the activity towards the tested fabrics was determined directly by extracting the remaining oils in the fabrics and further GC analysis (IST-ID), Polur1 showed good results for 61988 (with 55% of the original concentration of extracted compounds present in the final textile vs. 80% in control), 67007 (36% vs. 63% in control), and E03130 (48% vs 91% in control) (Annex Fig. 33).
Detailed methodology of activity assay:	Assay conditions at CSIC: 1) Using a circle hole puncher, a small piece (5mm diameter, 4 mg) of each of the fabrics are cut [3X58, E031303, 61988F1, 5237-00, 61488F1 and 67007]; 2) The small piece is added to a 2-ml safe-lock Eppendorf® polypropylene tubes (ref. 0030 120.094, Greiner Bio-One GmbH, Kremsmünster, Austria); 3) Then, 100 µl of 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer at pH 7.0 are added; and 4) The appropriated amount of enzyme is added, and the reaction is maintained under the set-up experimental conditions, after which the release of fatty acids is determined by a colorimetric method using the NEFA-Kit (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany), as follows: in a 96 well plate, 10 µl of the reaction solution + 100 µl of NEFA solution 1 (R1a) are transferred. Following 6 min incubation at 37°C, 50 µl of NEFA solution 2 (R2a) are added. After 6 min incubation at 30°C, the samples' absorbance is measured at 550nm using a Synergy HT Multi Mode Microplate Reader (Agilent, Madrid, Spain).

	Assay Conditions at IST-ID: 30 ºC; pH 8; Tris-HCl buffer at 40 mM; Untreated textiles 61488, 61988, 67007, 3X58 and E03130, cut into squares with approx. 25 mm^2 (from 8 to 43 mg, depending on the fabrics) Enzyme concentration, in reaction media, of 0.075 g L^{-1}. Testing Procedure: Untreated textiles were first cut and weighed. Each piece of textile was placed in a 1.5 mL microtubes with 400 µL of Tris-HCl buffer at 40 mM, pH 8. 20 µL of enzyme solution at 1.5 mg mL^{-1} (prepared with Tris-HCl buffer at 40 mM) were then added to each tube; no enzymes were added to two replicas of each textile, as a control. Tubes were incubated for 65 h, at 30 º, in an orbital shaker at 200 rpm. After incubation, fatty acid concentration in the reaction media were evaluated with NEFA-HR(2) kit, according to standard procedure. Additionally, textiles were further processed for quantification of fatty acids with GC-MS. Textiles were transferred to new microtubes, and dried at 40 ºC at low pressure. Dried textiles were than subjected to instant FAME procedure (MIDI Inc.); the resulting methylated fatty acids, and other compounds that were extracted, were analyzed by GC-MS; quantification of the fatty acids was performed based on an external standard curve. Untreated textiles and pre-treated textiles were also subjected to the same extraction and analytical procedure
Verified positive and negative controls for the reaction:	Positive control: none Negative control: 10-40 mM HEPES, K_2HPO_4 buffer (pH 7.0), or Tris-HCl buffer (pH 8.0).

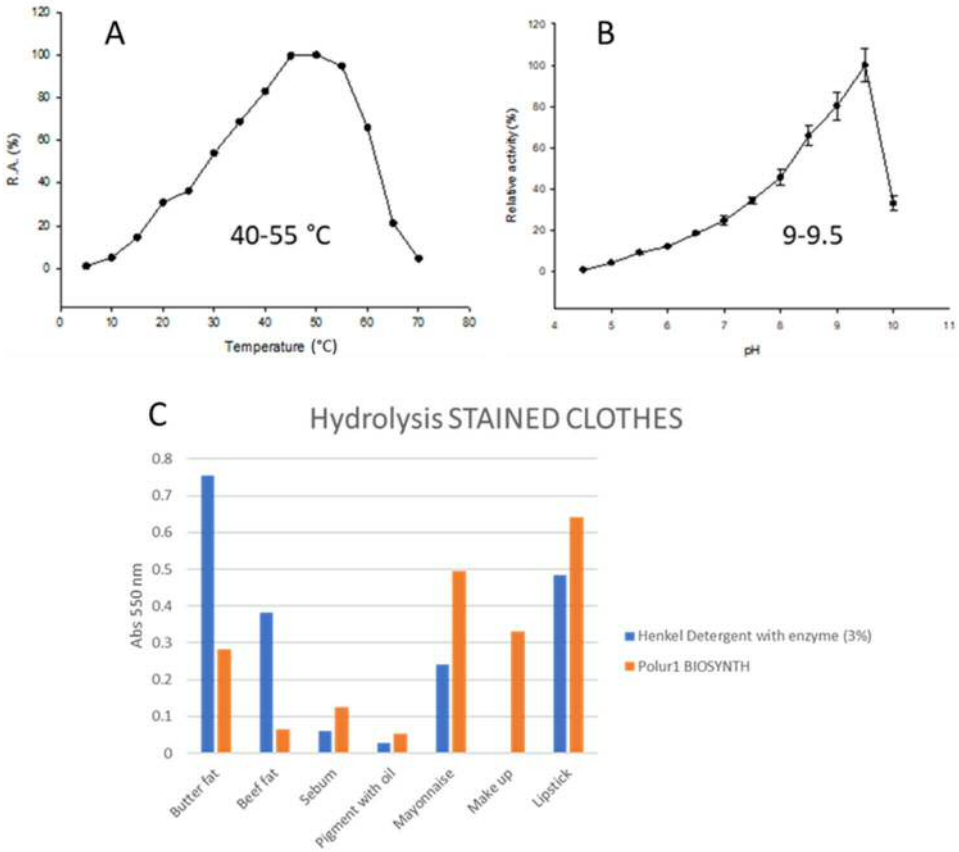
Tested range of conditions:			
Temperature [°C]:	5-70°C (Annex Fig. 29A)	Substrate:	Schoeller fabrics 3X58, E031303, 61988F1, 5237-00, 61488F1, 67007.
pH [-]:	4.5 - 10.0 (Annex Fig. 29B)	Substrate concentration [g_T/L]:	6.6-86 g_T/L (depending on the fabrics)
Medium (water, buffer, salt, cofactors, etc.):	HEPES: 10-40 mM K_2HPO_4: 10-40 mM Tris-HCl: 40 mM	Expected product(s) and their quantities:	Oil-free textile (solid), glycerol (soluble), free fatty acids (soluble); exact composition unknown
Time / duration:	2 – 65 h	Analytical methods:	Free fatty acids: Colorimetric method using the NEFA-Kit (indirect method) or GC (direct method).
Enzyme concentration [unit]:	0.01 – 0.9 g_E/g_T	Other remarks:	Only when small amount of enzymes are tested (e.g., lower than 0.01 g_E/g_T), long incubation times (e.g., 65 h) are needed.

Attachment (additional figures, descriptions, etc.)

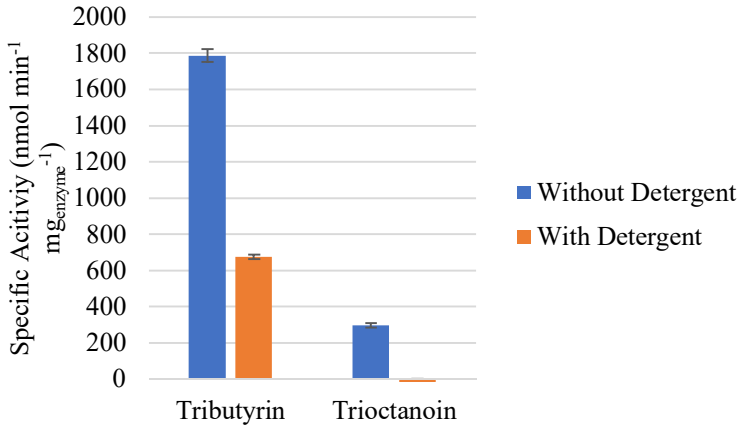
Polur1 is an enzyme with lipase activity. It has a molecular weight of 64.46 kDa. Its maximum activity is achieved at a temperature of 50°C, retaining over 80% of its activity between the temperature range of 40-55°C (Annex Fig. 29A). Additionally, it displays an alkaline optimal pH level, specifically at 9.5, while preserving more than 80% of its activity between the pH range of 9-9.5 (Annex Fig. 29B).

Besides, Polur1 B01 hydrolyses from short-chain to long-chain esters (not shown). It also shows higher activity for some stained clothes compared to HENKEL® Liquid Laundry Detergent_A with enzymes (Annex Fig. 29C), and this is why we recommend using it as detergent additive. The application conditions for detergent applications of Polur1 are compatible with the fact that it retains activity and even becomes activated in the presence of washing liquor with a model substrate like tributyrin (Annex Fig. 30), as determined by IST-ID using Polur1 B01. That said, the activity with trioctanoin significantly dropped. When Polur1 was tested for detergent applications using a buffer instead of washing liquour, IST-ID found that it showed better activity at 30 °C for all stains except lipstick and sebum (Annex Fig. 31). Under the tested conditions (30°C, Tris buffer 40 mM, pH 8.0), Polur 1 was most effective for mayo stains (Annex Fig. 32).

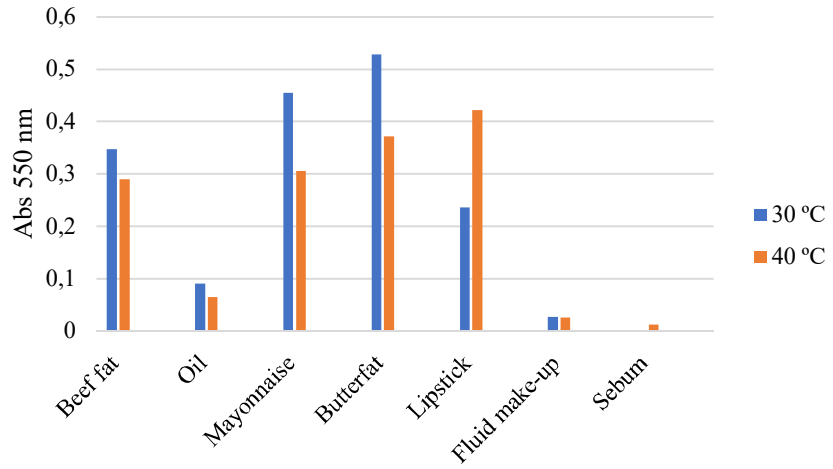
Polur1 lipase (B01) can be used for spinning oil removal in Schoeller fabrics, although it has low activity towards the tested fabrics when removal was determined indirectly by measuring the release of fatty acids to the reaction media as detected with the NEFA-kit (data not shown). However, when the activity towards the tested fabrics was determined directly by extracting the remaining oils in the fabrics and further GC analysis (IST-ID), Polur1 showed good results for 61988 (with 55% of the original concentration of extracted compounds present in the final textile vs. 80% in control), 67007 (36% vs. 63% in control), and E03130 (48% vs 91% in control) (Annex Fig. 33).



Annex Fig. 29. Characteristics of Polur1 and efficiency for detergent applications. A. Thermal profile of Polur1 B01. The data represents the relative percentages (%) of specific activity (U/mg) compared with the maximum activity using tripropionin (100 mM) as substrate. B. pH profile of Polur1 B01. The data represents the relative percentages (%) of specific activity (U/mg) compared with the maximum activity using p-nitrophenyl butyrate (pNPC4) as substrate. C. Absorbance values measured at 550 nm, of free fatty acids released from the different stained clothes under tests conditions.

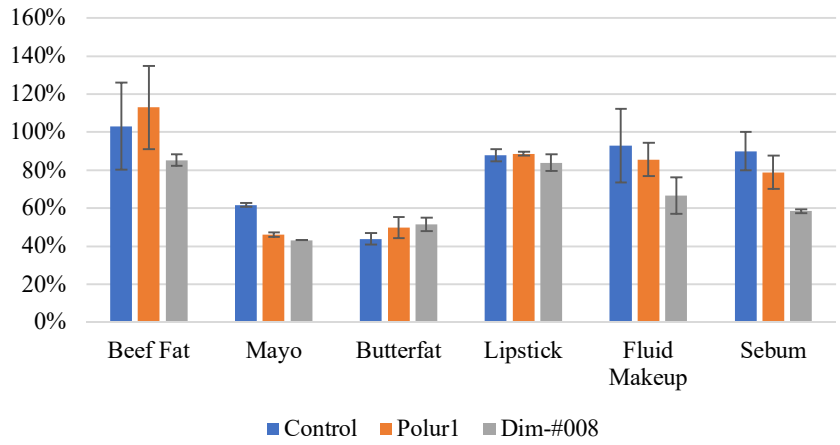


Annex Fig. 30. Specific activity of Polur1 B01 with tributyrin and trioctanoin, with and without detergent. Error bars represent standard deviation. Assay conditions: 30°C; pH 8; EPPS buffer at 2.5 mM; tributyrin or trioctanoin as substrate, at 4.545 mM; Lip9 from Biosynth concentration, in reaction media, of 0.227 g L^{-1}; With/Without 3.1 g L^{-1} detergent (washing liquour). A pH shift assay based on the indicator phenol red was used in these assays. Briefly: 1) In 96 microtiter plates, the following solutions were added sequentially: 90.9 µL of EPPS buffer at 2.5 mM, pH 8, with or without detergent A at 3.1 g L^{-1}; 9.09 µL substrate, at 100 mM, either tributyrin or trioctanoin, in DMSO; 90.9 µL phenol red at 0,304 mM, prepared in EPPS buffer, with or without detergent; 9.09 µL enzyme solution, at 0.5 g L^{-1}, prepared in EPPS buffer at 2.5 mM. 2) Absorbance at 550 nm was measured for 45 min, at 30 °C of incubation, with periodic shaking of the plate, using a Thermo Scientific Multiskan GO. Activity is determined by the variation of absorbance over time.

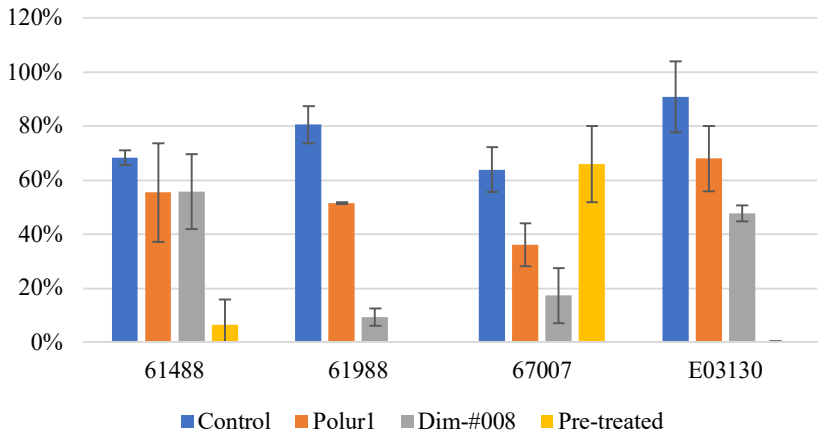


Annex Fig. 31. Polur1 B01-Results for stain activity screening, representing absorbance results from NEFA-HR(2). Bars represent average of 2 replicas. Assay Conditions: 30 °C/40 °C; pH 8; Tris-HCl buffer at 40 mM; Stains tested: Pigment with oil on polyester/cotton (PC-09), Mayonnaise on cotton (C-S-05S), 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), cut into disks with 6 mm in diameter; Enzyme concentration, in reaction media, of 0.075 g L-1. Testing Procedure: Disks, with a diameter of 6 mm, of standard stains were first cut with a hole puncher and weighed. Each disk placed in a 1.5 mL microtubes with 200 µL of Tris-HCl buffer at 40 mM, pH 8. 10 µL of enzyme solution at 1.5 mg mL-1 (prepared with Tris-HCl buffer at 40 mM) were then added to each tube; no enzymes were added to two replicas of each stain, as a control. Tubes were incubated

for 24 h, at either 30 °C or 40 °C, in an orbital shaker at 200 rpm. After incubation, fatty acid concentration in the reaction media were evaluated with NEFA-HR(2) kit, according to standard procedure.



Annex Fig. 32. Total concentration of extracted compounds from stained cloths after assay, determined by GC-MS. Resuls are presented as percentage of the concentration of the original stains. Error bars represent standard deviation. B01-Results for stain activity screening, representing absorbance results from NEFA-HR(2). Bars represent average of 2 replicas. Assay Conditions: 30 °C/40 °C; pH 8; Tris-HCl buffer at 40 mM; Stains 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), cut into disks with 6 mm in diameter; Enzyme concentration, in reaction media, of 0.075 g L-1. Testing Procedure: Disks, with a diameter of 6 mm, of standard stains were first cut with a hole puncher and weighed. Each disk placed in a 1.5 mL microtubes with 200 µL of Tris-HCl buffer at 40 mM, pH 8. 10 µL of enzyme solution at 1.5 mg mL-1 (prepared with Tris-HCl buffer at 40 mM) were then added to each tube; no enzymes were added to two replicas of each stain, as a control. Tubes were incubated for 24 h, at either 40 °C, in an orbital shaker at 200 rpm. After incubation, stain disks were further processed for quantification of fatty acids with GC-MS. Stain disks were transferred to new microtubes, and dried at 40 °C at low pressure. Dried disks were than subjected to instant FAME procedure (MIDI Inc.); the resulting methylated fatty acids, and other compounds that were extracted, were analyzed by GC-MS; quantification of the fatty acids was performed based on an external standard curve. Untreated stains were also subjected to the same extraction and analytical procedure.



Annex Fig. 33. Total concentration of extracted compounds from textiles after assay with Polur1 B01, determined by GC-MS, and of pre-treated textiles provided by Schoeller, presented as percentage of concentration of original textiles. Error bars represent standard deviation. As control, lipase Dim#008 is also present. Assay Conditions: 30 °C; pH 8; Tris-HCl buffer at 40 mM; Untreated textiles 61488, 61988, 67007, 3X58 and E03130, cut into squares with approx. 25 mm^2; Enzyme concentration, in reaction media, of 0.075 g L^{-1}. Testing Procedure: Untreated textiles were first cut and weighed. Each piece of textile was placed in a 1.5 mL microtubes with 400 µL of Tris-HCl buffer at 40 mM, pH 8. 20 µL of enzyme solution at 1.5 mg mL^{-1} (prepared with Tris-HCl buffer at 40 mM) were then added to each tube; no enzymes were added to two replicas of each textile, as a control. Tubes were incubated for 65 h, at 30 °, in an orbital shaker at 200 rpm. After incubation, fatty acid concentration in the reaction media were evaluated with NEFA-HR(2) kit, according to standard procedure. Additionally, textiles were further processed for quantification of fatty acids with GC-MS. Textiles were transferred to new microtubes, and dried at 40 °C at low pressure. Dried textiles were than subjected to instant FAME procedure (MIDI Inc.); the resulting methylated fatty acids, and other compounds that were extracted, were analyzed by GC-MS; quantification of the fatty acids was performed based on an external standard curve. Untreated textiles and pre-treated textiles were also subjected to the same extraction and analytical procedure.

Additional information is shown in Supplementary Material (Supplementary Fig. 10).

Enzyme specification sheet

Original version:	[date]15.01.2024 14:05:00	CSIC	Paula Vidal	p.vidal.ramon@csic.es
Last update:	[date]	CSIC	Paula Vidal	p.vidal.ramon@csic.es

Enzyme:	**#10**	**FE_EH37**
Enzyme class:	List of enyzme preparations (quantities for industrial tests have already been shared with partners):	Target application sector(s):
choose or enter	1. [B01] | BIOSYNTH |Pp_ExCel (Lot: 03919423SS1116)	☒ Detergents ☐ Textiles (specify) ☐ Oil removal ☐ Dye removal ☐ Cosmetics ☐ Other:_____

Enzyme origin					
Identifying partner:	CSIC	Type of expression:	Intracellular	Mode of application:	Whole cell
Original host:	*E. coli*	Purification and formulation:	Cell-free (liquid) [enzyme fraction]	Measured activity [U/mg]:	Glyceryl tripropionate: 2.0 U/mg
Further characteristics / comments:	[text]				

Production batch: [B01]					
Producing partner:	Biosynth	Type of expression:	Secreted	Mode of application:	Cell extract (unpurified)
Production host:	*P. pastoris*	Purification and formulation:	Lyophylised Supernatant	Measured activity:	Glyceryl tripropionate: 0.46 U/mg
Further characteristics / comments:	[text]				

Production batch: [Batch number]					
Producing partner:	[institution]	Type of expression:	Choose	Mode of application:	Choose
Production host:	[host organism]	Purification and formulation:	[formulation] [enzyme fraction]	Measured activity:	[text]
Further characteristics / comments:	[text]				

Application 1: Detergents

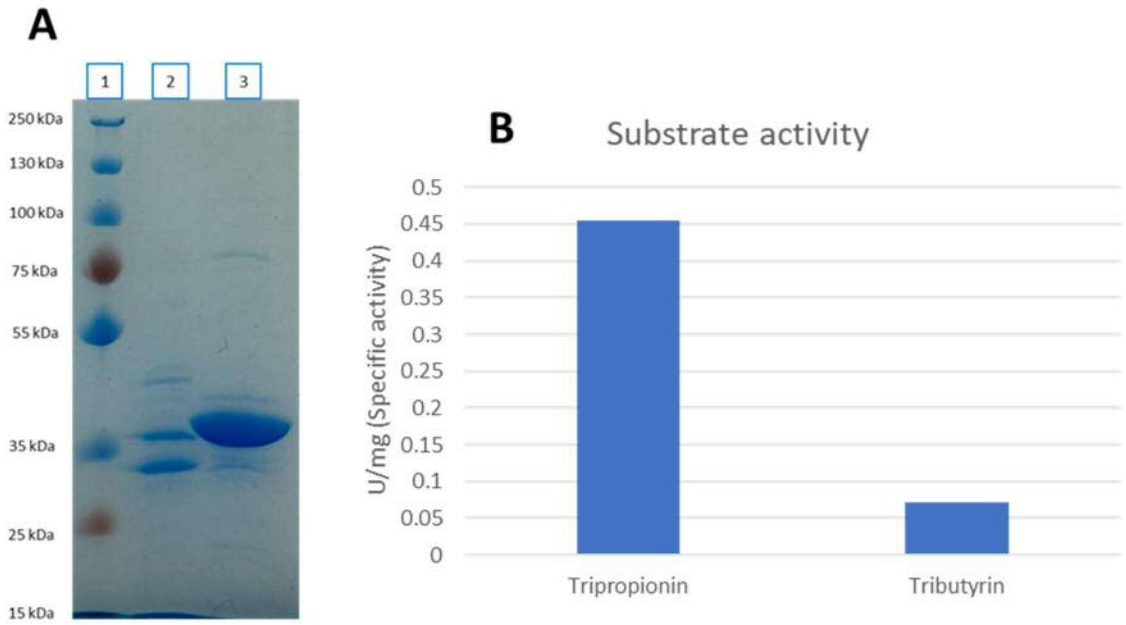
Recommended application conditions (from research perspective):			
Temperature [°C]*:	30 °C	Substrate:	-Tripropionine, tributyrin -Detergents: Stained clothes PC-09, C-S-05S, P-S-16, C-S-17, PC-S-132, C-S-61, C-S-10.
pH [-]*:	7.0	Substrate concentration [g/L]: [g of cloth per L of buffer solution]	-Tripropionine, tributyrin (9mM) -Detergents: 40 g/L
Medium (water, buffer, salt, cofactors, etc.)*:	40 mM HEPES buffer pH 7.0 Detergents: Washing liquor (HENKEL®).	Expected product(s) and their quantities:	Unknown (Free fatty acid)
Time / duration:	66h detergents	Analytical methods:	Colorimetric method using Phenol red for tripropionin and tributyrin and NEFA-kit for stained clothes
Enzyme concentration [mg/ml]: [mg of lyophilizate per ml of buffer solution]	-0.45 mg/ml for tributyrin and tripropionin -Detergents: 0.9 mg/ml	Other remarks on the set-up of the enzyme reaction:	
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

Brief description of observed activity (under recommended conditions):	We did not observe activity towards any of the HENKEL clothes.
Detailed methodology of activity assay:	Our small-scale assays and analytics are set-up as follows: 1) Using a circle hole puncher, a small piece (5mm diameter, 4 mg) of each of the stained clothes or fabrics are cut [stained clothes include 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); fabrics include, 3X58, E031303, 61988F1, 5237-00, 61488F1 and 67007]; 2) The small piece is added to a 2-ml safe-lock Eppendorf® polypropylene tubes (ref. 0030 120.094, Greiner Bio-One GmbH, Kremsmünster, Austria); 3) Then, 100 µl of 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer at pH 7.0 are added; and 4) The appropiated amount of enzyme is added, and the reaction is maintained under the set-up experimental conditions, after which the release of fatty acids is determined by a colorimetric method using the NEFA-Kit (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany), as follows: in a 96 well plate, 10 µl of the reaction solution + 100 µl of NEFA solution 1 (R1a) are transferred. Following 6 min incubation at 37°C, 50 µl of NEFA solution 2 (R2a) are added. After 6 min incubation at 30°C, the samples' absorbance is measured at 550nm using a Synergy HT Multi Mode Microplate Reader (Agilent, Madrid, Spain).
Verified positive and negative controls for the reaction:	

Tested range of conditions:

Temperature [°C]:	30 °C	Substrate:	Tripropionine, tributyrin Detergents: Stained clothes PC-09, C-S-05S, P-S-16, C-S-17, PC-S-132, C-S-61, C-S-10.
pH [-]:	7.0	Substrate concentration [g/L]: [g of cloth per L of buffer solution]	-Tripropionine, tributyrin (9mM) -Detergents: 40 g/L
Medium (water, buffer, salt, cofactors, etc.):	40 mM HEPES buffer pH 7.0 Detergents: Washing liquor (HENKEL®).	Expected product(s) and their quantities:	Unknown (Free fatty acid)
Time / duration:	66h detergents	Analytical methods:	Colorimetric method using Phenol red for tripropionin and tributyrin and NEFA-kit for stained clothes
Enzyme concentration [mg/ml]: [mg of lyophilizate per ml of buffer solution]	-0.45 mg/ml for tributyrin and tripropionin -Detergents: 0.9 mg/ml	Other remarks:	

Attachment (additional figures, descriptions, etc.)



Annex Fig. 34. Characteristics of EH37. **A**. SDS-PAGE analysis. A 12% SDS-polyacrylamide gel is shown. Lane 1. Molecular weight marker. Lane2. EH37 produced by Biosynth in *Pichia* Lane3. EH37 CSIC pure enzyme produced in *E. coli*. **B**. Specific activity (U/mg) of EH37 from Biosynth with tripropionin and tributyrin at pH 7.0 and 30°C.

Additional information is shown in Supplementary Material (Supplementary Fig. 4).

Enzyme specification sheet

Original version:	23.10.2023	CSIC	Paula Vidal	p.vidal.ramon@csic.es
Last update:	28.10.2023	CSIC	Paula Vidal	p.vidal.ramon@csic.es

Enzyme:	**#11**	**FE_Lip9**	
Enzyme class:	List of enyzme preparations (quantities for industrial tests have already been shared with partners):		Target application sector(s):
Esterase / Lipase / PETase	1. B01 | CSIC | Ec_InCel_His_pur (Lot: 11.09.2023) 2. B02 | BIOSYNTH | Pp_ExCel (Lot: 03908223SS0516) 3. B03 | CSIC | Ec_InCel_extr (Lot: 08.11.2022)		☒ Detergents ☒ Textiles (specify) ☒ Oil removal ☐ Dye removal ☐ Cosmetics ☐ Other:_____

Enzyme origin					
Identifying partner:	CSIC	Type of expression:	Intracellular	Tested enzyme fraction:	Soluble enzyme
Original host:	*E. coli* BL21	Purification and formulation:	[formulation] [enyzme fraction]	Measured activity [unit]:	[text]
Further characteristics / comments:	[text]				

Production batch: B01					
Producing partner:	CSIC	Type of expression:	Intracellular	Mode of application:	Soluble enzyme
Production host:	*E. coli* BL21	Purification and formulation:	Lyophylised Extract	Measured activity:	Glyceryl tributirate: 2.5 U/mg
Further characteristics / comments:	Purified enzyme (His6-tag); activity measured for tributyrin (30°C, pH 8.0)				

Production batch: B02					
Producing partner:	Biosynth	Type of expression:	Secreted	Mode of application:	Soluble enzyme
Production host:	*P. pastoris*	Purification and formulation:	Lyophylised Supernatant	Measured activity:	Glyceryl tributirate: 0.21 U/mg
Further characteristics / comments:	Activity measured for tributyrin (30°C, pH 8.0)				

Production batch: B03					
Producing partner:	CSIC	Type of expression:	Intracellular	Mode of application:	Soluble enzyme
Production host:	*E. coli* BL21	Purification and formulation:	Lyophylised Extract	Measured activity:	Glyceryl tributirate: 0.43 U/mg
Further characteristics / comments:	Unpurified intracellular protein extract; activity measured for tributyrin (30°C, pH 8.0)				

Application 1: Detergents

Recommended application conditions (from research perspective):			
Temperature [°C]*:	30 °C (optimal) 30-40°C (acceptable) (Annex Fig. 35A,B)	Substrate:	Stained clothes PC-09, C-S-05S, P-S-16, C-S-17, PC-S-132, C-S-61, C-S-10
pH [-]*:	7.0 (optimal) 7.0-10.0 (acceptable) (Annex Fig. 35C)	Substrate concentration [g_C/L]: Grams of stained clothes per L of wash liquor	40 g_C/L
Medium (water, buffer, salt, cofactors, etc.)*:	Washing liquor (HENKEL®) (2.5 g/L)	Expected product(s) and their quantities: Grams of enzyme per L of wash liquor	Fat-free clothes (solid), glycerol (soluble), free fatty acids (soluble); exact composition unknown
Time / duration:	30 min	Analytical methods:	Free fatty acids: Colorimetric method using the NEFA-Kit.
Enzyme concentration [g_E/L]: Grams of enzyme per L of wash liquor	B01: 5 g_E/L B02: 50 g_E/L B03: 50 g_E/L	Other remarks on the set-up of the enzyme reaction:	Discuss with Henkel if measurement of the "cleanliness" of the clothes is required
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

Brief description of observed activity (under recommended conditions):	Lip9 pure CSIC lipase (B01) shows high activity for long chain triglycerides (see Annex Fig. 35G). For detergent, B01 shows higher activity for some stained clothes compared to HENKEL® Liquid Laundry Detergent_A with enzymes (Annex Fig. 35H). The amount of lipase Lip9 in sample B02 is relatively low (Annex Fig. 35I). This is why B02 can be tested under conditions of B01, but it is approximately 10 times less active. Lip9 B03 can be tested under conditions of B01, but it is approximately 2 times less active than B01. This is why we recommend using 10-times higher amount of B02 and B03 compared to B01 for the detergent applications. It is to highlight that the application conditions for detergent applications are compatible with the enzyme's stability in the presence of washing liquor at 30°C (Annex Fig. 36; T_{50} at 30°C being 2.6 ± 0.1 h) and the fact that it retains activity and even becomes activated in the presence of washing liquor with a model substrate like tributyrin (Annex Fig. 37).
Detailed methodology of activity assay:	Our small-scale assays and analytics are set-up as follows: 1) Using a circle hole puncher, a small piece (5 mm diameter, 4 mg) of each of the stained clothes are cut [stained clothes include 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); 2) The small piece is added to a 2-ml safe-lock Eppendorf® polypropylene tubes (ref. 0030 120.094, Greiner Bio-One GmbH, Kremsmünster, Austria); 3) Then, 100 µl of washing liquor (2.5 g/L) are added; and 4) The appropriated amount of enzyme is added, and the reaction is maintained under the set-up experimental conditions, after which the release of fatty acids is determined by a colorimetric method using the NEFA-Kit (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany), as follows: in a 96 well plate, 10 µl of the reaction solution + 100 µl of NEFA solution 1 (R1a) are transferred. Following 6 min incubation at 37°C, 50 µl of NEFA solution 2 (R2a) are added. After 6 min incubation at

	30°C, the samples' absorbance is measured at 550nm using a Synergy HT Multi Mode Microplate Reader (Agilent, Madrid, Spain).
Verified positive and negative controls for the reaction:	Positive control: HENKEL® Liquid Laundry Detergent_A including enzymes. Negative control: Washing liquor buffer (without enzymes)

Tested range of conditions:			
Temperature [°C]:	5-70°C (Annex Fig. 35A,B)	Substrate:	Stained clothes PC-09, C-S-05S, P-S-16, C-S-17, PC-S-132, C-S-61, C-S-10.
pH [-]:	4.0 - 11.0 (Annex Fig. 35C)	Substrate concentration [g_C/L]:	40 g_C/L
Medium (water, buffer, salt, cofactors, etc.):	Washing liquor (HENKEL®) (2.5 g/L)	Expected product(s) and their quantities:	Fat-free clothes (solid), glycerol (soluble), free fatty acids (soluble); exact composition unknown
Time / duration:	0.5-4 h	Analytical methods:	Free fatty acids: Colorimetric method using the NEFA-Kit. No other method tested.
Enzyme concentration [g_E/L]:	2-50 g_E/L	Other remarks:	

Application 2: Textiles (Oil removal)

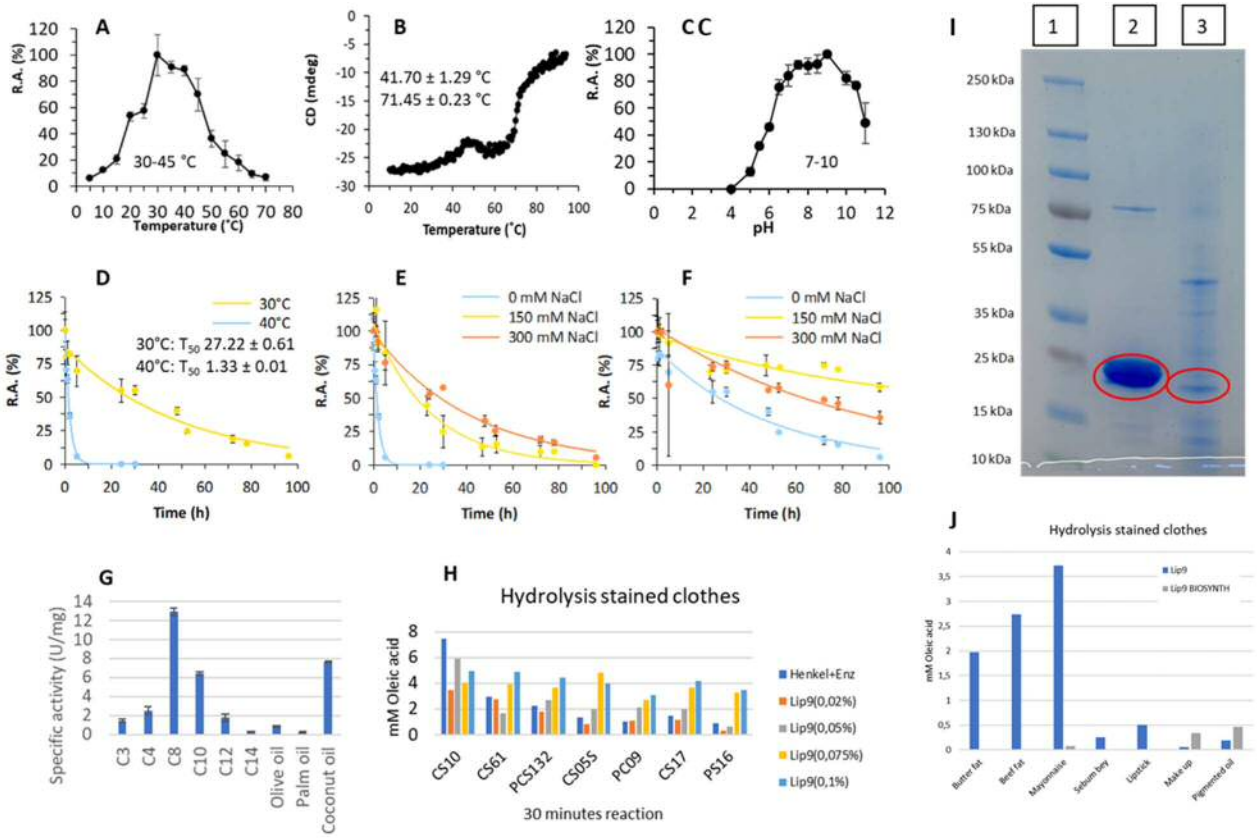
Recommended application conditions (from research perspective):			
Temperature [°C]*:	30 °C (optimal) 30-40°C (acceptable) (Annex Fig. 35A,B)	Substrate:	Schoeller fabrics 3X58, E031303, 61988F1, 5237-00, 61488F1, 67007 (all ROH fabrics). Raw textiles.
pH [-]*:	7.0 (optimal) 7.0-10.0 (acceptable) (Annex Fig. 35C)	Substrate concentration [g_T/L]: (Grams of oil-containing textile per L of buffer solution)	34 g_T/L
Medium (water, buffer, salt, cofactors, etc.)*:	10-40 mM HEPES or K_2HPO_4 buffer (pH 7.0) with 150 mM NaCl.	Expected product(s) and their quantities:	Oil-free textile (solid), glycerol (soluble), free fatty acids (soluble); exact composition unknown
Time / duration:	24 h	Analytical methods:	Free fatty acids: Colorimetric method using the NEFA-Kit.
Enzyme concentration [g_E/g_T]: (Grams of enzyme per gram of textile)	B01: 0.15 g_E/g_T B02: 1.5 g_E/g_T B03: 1.5 g_E/g_T	Other remarks on the set-up of the enzyme reaction:	
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

Brief description of observed activity (under recommended conditions):	Lip9 pure (B01) lipase shows high activity for long chain triglycerides (see Annex Fig. 35G). For textiles, Lip9 (B01) has low activity towards the tested fabrics. We used HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ref: H3375, Sigma Aldrich) buffer pH 7.0 plus 150 mM NaCl, but we think any other buffer (containing 150 mM NaCl) with neutral pH can be used. The addition of NaCl is highly recommended (see stability details in Annex Fig. 35D-F). The amount of lipase Lip9 in sample B02 is relatively low (Annex Fig. 35I). This is why B02 can be tested under conditions of B01, but it is approximately 10 times less active. Lip9 B03 can be tested under conditions of B01, but it is approximately 2 times less active than B01. This is why we recommend using 10-times higher amount of B02 and B03 compared to B01 for the textile applications.
Detailed methodology of activity assay:	Our small-scale assays and analytics are set-up as follows: 1) Using a circle hole puncher, a small piece (5mm diameter, 4 mg) of each of the fabrics are cut [3X58, E031303, 61988F1, 5237-00, 61488F1 and 67007]; 2) The small piece is added to a 2-ml safe-lock Eppendorf® polypropylene tubes (ref. 0030 120.094, Greiner Bio-One GmbH, Kremsmünster, Austria); 3) Then, 100 µl of 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer at pH 7.0 are added; and 4) The appropriated amount of enzyme is added, and the reaction is maintained under the set-up experimental conditions, after which the release of fatty acids is determined by a colorimetric method using the NEFA-Kit (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany), as follows: in a 96 well plate, 10 µl of the reaction solution + 100 µl of NEFA solution 1 (R1a) are transferred. Following 6 min incubation at 37°C, 50 µl of NEFA solution 2 (R2a) are added. After 6 min incubation at 30°C, the samples' absorbance is measured at 550nm using a Synergy HT Multi Mode Microplate Reader (Agilent, Madrid, Spain).
Verified positive and negative controls for the reaction:	Positive control: none Negative control: 10-40 mM HEPES or K_2HPO_4 buffer (pH 7.0) with 150 mM NaCl.

Tested range of conditions:			
Temperature [°C]:	5-70°C (Annex Fig. 35A,B)	Substrate:	Schoeller fabrics 3X58, E031303, 61988F1, 5237-00, 61488F1, 67007 (all ROH fabrics).
pH [-]:	4.0 - 11.0 (Annex Fig. 35C)	Substrate concentration [g_T/L]:	34 g_T/L
Medium (water, buffer, salt, cofactors, etc.):	NaCl: 0 – 300 mM HEPES: 10-40 mM K_2HPO_4: 10-40 mM	Expected product(s) and their quantities:	Oil-free textile (solid), glycerol (soluble), free fatty acids (soluble); exact composition unknown
Time / duration:	2 – 24 h	Analytical methods:	Free fatty acids: Colorimetric method using the NEFA-Kit.
Enzyme concentration [unit]:	0.06 – 1.5 g_E/g_T	Other remarks:	

Attachment (additional figures, descriptions, etc.)

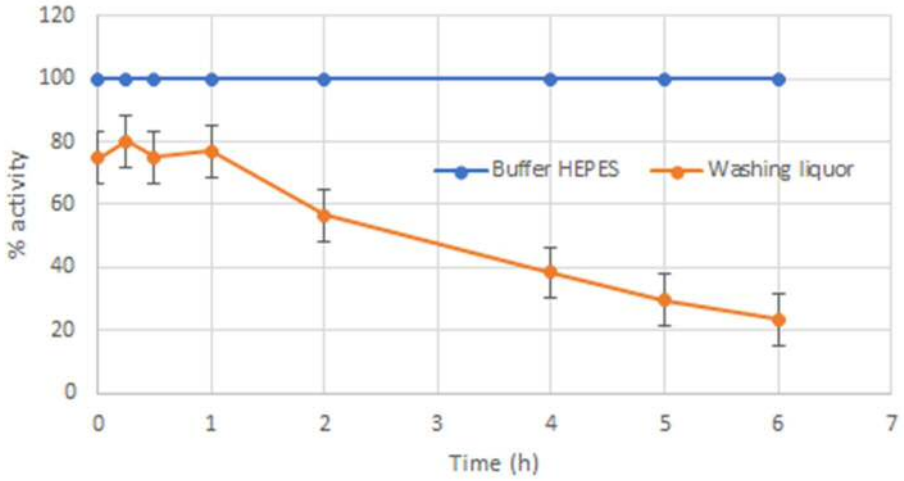
Lip9 is an enzyme with lipase and PETase properties. It has a molecular weight of 19.5 kDa. Its maximum activity is achieved at a temperature of 30°C, retaining over 80% of its activity between the temperature range of 30-40°C (Annex Fig. 35A) in consonance with its denaturing temperature at 41.7° (Annex Fig. 35B). Additionally, it displays an alkaline optimal pH level, specifically at 9.0, while preserving more than 80% of its activity between the pH range of 7.0-10.0 (Annex Fig. 35C). The stability, by meaning of half-life (T_{50}), of Lip9 at 30°C is about 27 h, and less than 1.3 h at 40°C (Annex Fig. 35D). However, the addition of 150 mM NaCl increased the T_{50} of Lip9 to 18.4 h at 40°C (Annex Fig. 35E) and to 154 h at 30°C (Annex Fig. 35F). This half-life is compatible with activity measurement assays conducted to assess triglyceride degradation (Annex Fig. 35G) and in detergent applications, where it is observed that Lip9 can clean stained clothes in 30 minutes at 30-40 °C (Annex Fig. 35H). However, since the amount of Lip9 is lower in preparation B02 compared to B01 (Annex Fig. 35I), the preparation B01 is 10 times more effective for cleaning than B02 (Annex Fig. 35J). The enzyme's half-life at 30°C in the absence (27 h) or presence (154 h) of NaCl is compatible with the 24-hour test period for textile applications. However, the enzyme's half-life at 40°C in the absence (1.3 h) or presence (18.4 h) of NaCl is shorter than the 24-hour test period for textile applications, which could result in the enzyme not being effective at 40°C in the absence of NaCl.



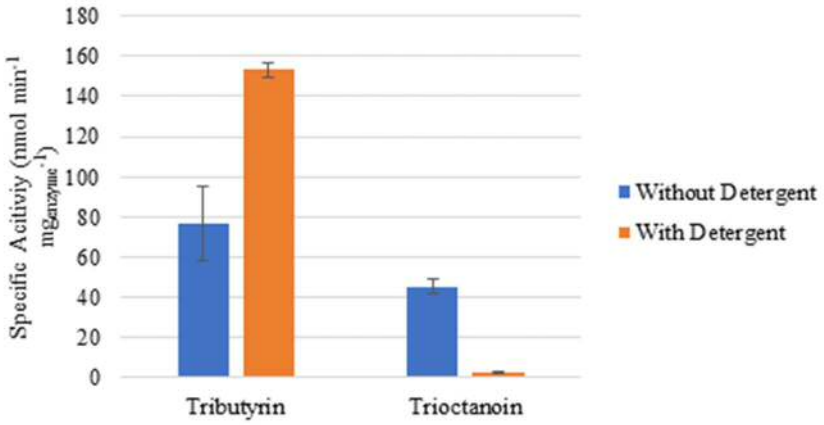
Annex Fig. 35. Characteristics of Lip9. **A.** Thermal profile of Lip9 (B01); the data represents the relative percentages (%) of specific activity (U/mg) compared with the maximum activity using tripropionin (100mM) as substrate. **B.** pH profile of Lip9; the data represents the relative percentages (%) of specific activity (U/mg) compared with the maximum activity using $pNPC_3$ as substrate. **C.** Denaturation temperature of Lip9 as determined by circular dichroism. **D.** Half-life (T50) time of Lip9 (B01)) at 30°C and 40°C; the data represents the relative activity percentages (%) of specific activity compared with the maximum activity using $pNPC_4$ as substrate, with T_{50} at 30°C and 40°C being 27.22 ± 0.61 and 1.33 ± 0.01 h, respectively. **E.** Half-life (T_{50}) time of Lip9 at 40°C; T_{50} at 40°C being 18.40 ± 0.21 h at 150 mM NaCl and 28.91 ± 0.29 h at 300 mM NaCl. **F.** Half-life (T_{50}) time of Lip9 at 30°C; T_{50} at 30°C being 154.1 ± 5.96 h at 150 mM NaCl and 63.63 ± 10.54 h at 300 mM NaCl. **G.** Specific activity (U/mg) of Lip9 (B01) with different esters at pH 7.0 and 30°C. **H.** Concentration (mM) of free fatty acids released by Lip9 (B01) from stained clothes under recommended application conditions. **I.** SDS-PAGE analysis. A 12% SDS-polyacrylamide gel is shown. Lane 1. Molecular weight marker. Lane 2. B01. Lane 3. B02. **J.** Concentration (mM) of free fatty acids

released from stained clothes by Lip9 B01 (Lip9) and B02 (Lip9 BIOSYNTH), under recommended application conditions.

The application conditions for detergent applications of Lip9 are compatible with the enzyme's stability in the presence of washing liquor at 30°C (Annex Fig. 36); in details, the half-life of Lip9 B01 in the presence of washing liquor at 30°C is 2.6 ± 0.1 h, that is 10-times lower than without washing liquour (27 h at 30ºC in HEPES buffer pH 7.0; see Annex Fig. 35D). The conditions are also compatible with the fact that it retains activity and even becomes activated in the presence of washing liquor with a model substrate like tributyrin (Annex Fig. 37), as determined by IST-ID using Lip9 B02. That said, the activity with trioctanoin significantly dropped; this may be due to the low activity of Lip9 B02 preparation rather than a problem of stability.



Annex Fig. 36. Half-life (T50) time of Lip9 (B01) at 30°C in the presence of washing liquour; the data represents the relative activity percentages (%) of specific activity compared with the maximum activity using $pNPC_4$ as substrate, with T_{50} at 30°C being 2.6 ± 0.1 h. For comparison, 40 mM HEPES buffer pH 7.0 was used.



Annex Fig. 37. Specific activity of Lip 9 B02 with tributyrin and trioctanoin, with and without detergent. Error bars represent standard deviation. Assay conditions: 30ºC; pH 8; EPPS buffer at 2.5 mM; tributyrin or trioctanoin as substrate, at 4.545 mM; Lip9 from Biosynth concentration, in reaction media, of 0.227 g L^{-1}; With/Without 3.1 g L^{-1} detergent (washing liquour). A pH shift assay based on the indicator phenol red was used in these assays. Briefly: 1) In 96 microtiter plates, the following solutions were added sequentially: 90.9 µL of EPPS buffer at 2.5 mM, pH 8, with or without detergent A at 3.1 g L^{-1}; 9.09 µL substrate, at 100 mM, either tributyrin or trioctanoin, in DMSO; 90.9 µL phenol red at 0,304 mM, prepared in EPPS buffer, with or without detergent; 9.09 µL enzyme solution, at 0.5 g L^{-1}, prepared in EPPS buffer at 2.5 mM. 2) Absorbance at 550 nm was measured for 45 min, at 30 ºC of incubation, with periodic shaking of the plate, using a Thermo Scientific Multiskan GO. Activity is determined by the variation of absorbance over time.

Additional information is shown in Supplementary Material (Supplementary Fig. 3, 10).

Enzyme specification sheet

Original version:	[date]12.01.2024 14:05:00	CSIC	Paula Vidal	p.vidal.ramon@csic.es
Last update:	[date]	CSIC	Paula Vidal	p.vidal.ramon@csic.es

Enzyme:	**#12**	**Hyal_HRDSV_2334**
Enzyme class:	List of enyzme preparations (quantities for industrial tests have already been shared with partners):	Target application sector(s):
choose or enter	1. [B01] | BIOSYNTH |Pp_ExCel (Lot: 03920323SS1127)	☐ Detergents ☐ Textiles (specify) ☐ Oil removal ☐ Dye removal ☒ Cosmetics ☐ Other:_____

Enzyme origin					
Identifying partner:	CSIC	Type of expression:	Intracellular	Mode of application:	Choose
Original host:	[host organism]	Purification and formulation:	[formulation] [enyzme fraction]	Measured activity [unit]:	[text]
Further characteristics / comments:	[text]				

Production batch: [03920323SS1127]					
Producing partner:	Biosynth	Type of expression:	Secreted	Mode of application:	Cell extract (unpurified)
Production host:	*P. pastoris*	Purification and formulation:	Lyophylised Supernatant	Measured activity:	HA: 0.99 U/g
Further characteristics / comments:	[text]				

Production batch: [Batch number]					
Producing partner:	[institution]	Type of expression:	Choose	Mode of application:	Choose
Production host:	[host organism]	Purification and formulation:	[formulation] [enyzme fraction]	Measured activity:	[text]
Further characteristics / comments:	[text]				

Application 1: Cosmetics

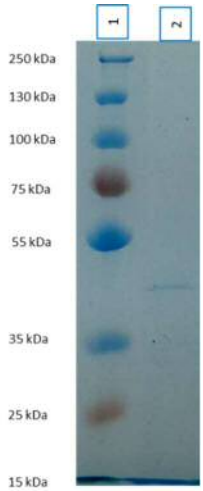
Recommended application conditions (from research perspective):			
Temperature [°C]*:	37 °C	Substrate:	HYACARE (Evonic) HYACARE 50 (Evonic) p-nitrophenol sugars
pH [-]*:	7.0	Substrate concentration [mg/ml]: [mg of substrate per ml of buffer solution]	HYACARE → 1mg/ml HYACARE 50 → 2mg/ml p-nitrophenols → 0.33 mg/ml
Medium (water, buffer, salt, cofactors, etc.)*:	40mM HEPES buffer (150 mM, 300mM NaCl, pH 6.0, 7.0 and 8.0)	Expected product(s) and their quantities:	Reduced sugars
Time / duration:	24h	Analytical methods:	BCA, *p*-nitrophenol assay and DNS assay
Enzyme concentration [mg/ml]: [mg of lyophilizate per ml of buffer solution]	3 mg/ml	Other remarks on the set-up of the enzyme reaction:	
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

Brief description of observed activity (under recommended conditions):	We did not observe activity towards HYACARE and HYACARE 50 substrates using BCA assay. Otherwise, Hyal_HRDSV_2334 seems to have activity against different types of p-nitrophenols sugars like: p-Nitrophenyl β-Glucopyranoside, p-Nitrophenyl β-Galactopyranoside, p-Nitrophenyl β-Xylopyranoside, p-Nitrophenyl β-Cellobioside, p-Nitrophenyl α-D-Fucopyranoside.
Detailed methodology of activity assay:	**BCA assay**: In a 2 ml Eppendorf we run the reaction using 180 µl of buffer solution, 3mg/ml of Hyal_HRDSV_2334 lyophilizate and 1mg/ml of HYACARE or 2mg/ml of HYACARE. We leave the reaction for 24 hours at 37°C 950 rpm. After this time, we add 150 µl of BCA assay solution to a 96 well plate into each well. Then we add 25µl of the sample to the appropriate well cover the plate and incubate at 70°C for 30 min: the colour formation will take place. Then we measure absorbance on spectrophotometer at 540 nm. ***p*-nitrophenol assay**: In a 96 well plate, we add 150 µl of HEPES 40 mM, pH 7.0 to each well, 0.33 mg/ml of each p-nitrophenol sugars (shown in Annex Fig. 39) and 2 mg/ml of Hyal_HRDSV_2334 lyophilizate. We leave the reaction at 37°C for 24 hours and after that time we stop the reaction by adding 50 µl of Na2CO3, 6%. **DNS assay:** In a 2 ml Eppendorf we run the reaction using 500 µl of buffer solution (HEPES 40 mM, pH 7.0), 20 mg/ml of Hyal_HRDSV_2334 lyophilizate and 20 mg/ml of each substrate (shown in Annex Fig. 40). We leave the reaction 30 minutes at 37°C 950 rpm. After this time, we add 50 µl of reaction and 50 µl of DNS solution to a 96 well plate into each well and we incubate it at 80°C during 25 minutes 200 rpm. After that, we let it cool for 5 minutes and measure the absorbance on spectrophotometer at 540 nm.
Verified positive and negative controls for the reaction:	BCA assay → Negative control: Only HA, only HA 50, only lyophilizate with buffer solution. p-nitrophenol assay → Negative control: Only p-nitrophenol substrate with buffer solution.

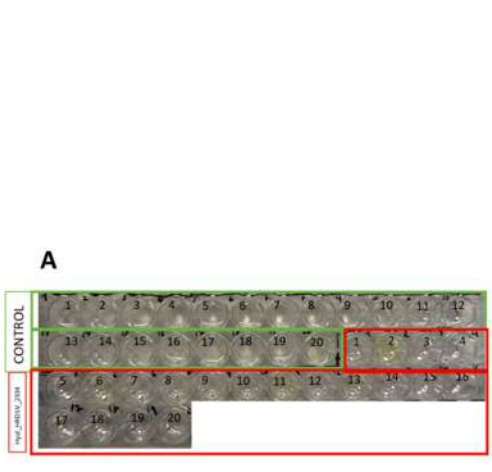
	DNS assay → Negative control: Only HA (Evonic), HA50 (Evonic), CMC, Avicel (MCC), Pollulan, Laminarin, Lichenin with buffer solution.

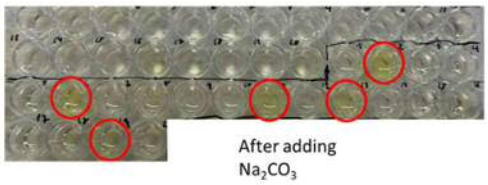
Tested range of conditions:			
Temperature [°C]:	37 °C	Substrate:	HYACARE (Evonic) HYACARE 50 (Evonic) p-nitrophenol sugars
pH [-]:	6.0, 7.0, 8.0	Substrate concentration [mg/ml]: [mg of substrate per ml of buffer solution]:	HYACARE → 1mg/ml HYACARE 50 → 2mg/ml *p*-nitrophenol sugars → 0.33 mg/ml
Medium (water, buffer, salt, cofactors, etc.):	40mM HEPES buffer (150 mM, 300mM NaCl, pH 6.0, 7.0 and 8.0)	Expected product(s) and their quantities:	Reduced sugars
Time / duration:	24h	Analytical methods:	BCA, p-nitrophenol assay and DNS assay.
Enzyme concentration [mg/ml]: [mg of lyophilizate per ml of buffer solution]	2-3 mg/ml	Other remarks:	

Attachment (additional figures, descriptions, etc.)



Annex Fig. 38. Characteristics of Hyal_HRDSV_2334**.** SDS-PAGE analysis. A 12% SDS-polyacrylamide gel is shown. Lane 1. Molecular weight marker. Lane 2. Hyal_HRDSV_2334 produced by Biosynth in *Pichia pastoris*.

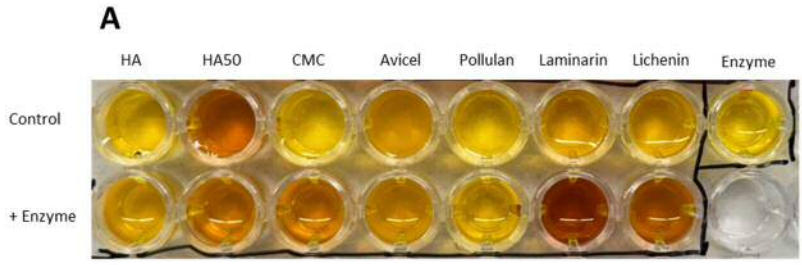




B

1) p-Nitrophenyl-α-Glucopyranoside
2) p-Nitrophenyl β-Glucopyranoside
3) p-Nitrophenyl α-Rhamnopyranoside
4) p-Nitrophenyl β-Lactoside
5) p-Nitrophenyl α-Galactopyranoside
6) p-Nitrophenyl β-Galactopyranoside
7) p-nitrophenyl α-L-arabinofuranoside
8) p-Nitrophenyl α-arabinopyranoside
9) p-Nitrophenyl β-arabinopyranoside
10) p-Nitrophenyl α-Xylopyranoside
11) p-Nitrophenyl β-Xylopyranoside
12) p-Nitrophenyl α-Maltoside
13) p-Nitrophenyl β-Cellobioside
14) p-Nitrophenyl β-Glucuronide
15) p-Nitrophenyl β-Mannopyranoside
16) p-nitrophenyl-N acetyl β-D-Glusocaminide
17) 4-nitrophenyl-N acetyl β-D-Galactosaminide
18) p-Nitrophenyl α-Mannopyranoside
19) p-Nitrophenyl α-D-Fucopyranoside
20) p-Nitrophenyl β-D-Fucopyranoside

Annex Fig. 39. *p*-Nitrophenol sugars assay with Hyal_HRDSV_2334. **A.** *p*-Nitrophenol assay plate. **B.** Name of all *p*-nitrophenol sugars used in the assay. In red the ones that Hyal_HRDSV_2334 are active with.



B

Substrate	U/g
HA	0.99
HA50	0
CMC	2.02
Avicel (MCC)	0
Pollulan	0
Laminarin	9.67
Lichenin	4.67

Annex Fig. 40. DNS Assay**. A.** DNS assay plate showing the coloring of the samples after measurement. **B**. Specific activity (U/g) of Hyal_HRDSV_2334 from Biosynth with different substrates; HA, HA50, CMC, Avicel (MCC), Pollulan, Laminarin and Lichenin.

Additional information is shown in Supplementary Material (Supplementary Fig. 7, 8).

Enzyme specification sheet

Original version:	31.01.2024	CSIC	Paula Vidal	p.vidal.ramon@csic.es
Last update:	31.01.2024	CSIC	Paula Vidal	p.vidal.ramon@csic.es

Enzyme:	**#13**	***Vibrio alginolyticus* #23**	
Enzyme class:	List of enyzme preparations (quantities for industrial tests have already been shared with partners):		Target application sector(s):
choose or enter	1. [B01] | Vibrio alginolyticus IAMC-CNR#23		☐ Detergents ☐ Textiles (specify) ☐ Oil removal ☐ Dye removal ☒ Cosmetics ☐ Other:_____

Enzyme origin					
Identifying partner:	CNR	Type of expression:	Intracellular	Mode of application:	Other (Lyophilizate and supernatant)
Original host:	*Vibrio alginolyticus* #23	Purification and formulation:	Lyophylised Supernatant	Measured activity [unit]:	-
Further characteristics / comments:	[text]				

Production batch: [B01]					
Producing partner:	BioC-CheM Solutions	Type of expression:	Intracellular	Mode of application:	Other (Lyophilizate and supernatant)
Production host:	*Vibrio alginolyticus* #23	Purification and formulation:	Lyophylised Supernatant	Measured activity:	-
Further characteristics / comments:	[text]				

Production batch: [Batch number]					
Producing partner:	[institution]	Type of expression:	Choose	Mode of application:	Choose
Production host:	[host organism]	Purification and formulation:	[formulation] [enyzme fraction]	Measured activity:	[text]
Further characteristics / comments:	[text]				

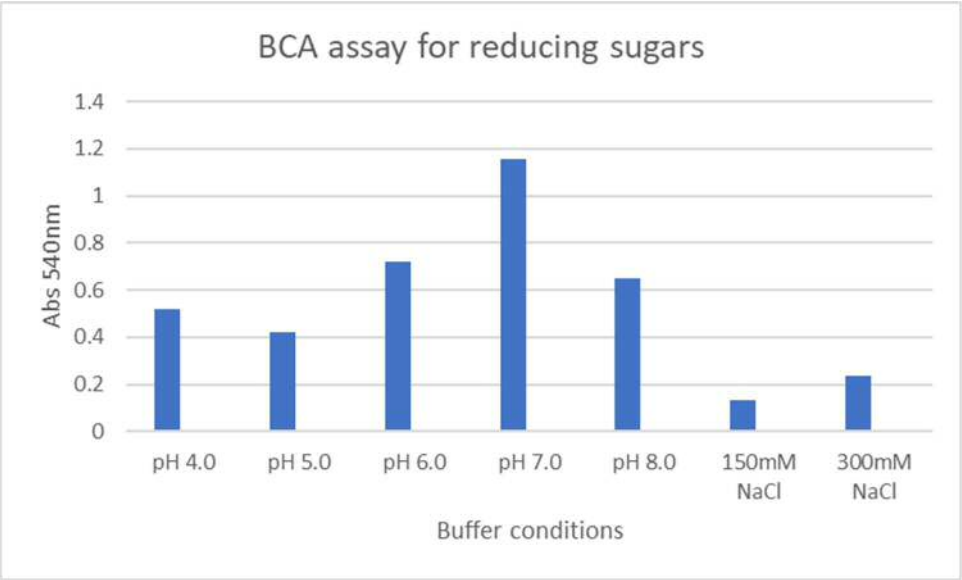
Application 1: Cosmetics

Recommended application conditions (from research perspective):			
Temperature [°C]*:	37°C	Substrate:	HYACARE, HA (Evonic) HYACARE 50, HA50 (Evonic)
pH [-]*:	7.0	Substrate concentration [mg/ml]: [mg of substrate per ml of buffer solution]	HA → 1mg/ml HA50 → 2mg/ml
Medium (water, buffer, salt, cofactors, etc.)*:	HEPES buffer 40mM, pH 7.0	Expected product(s) and their quantities:	Reduced sugars
Time / duration:	72 hours	Analytical methods:	BCA assay
Enzyme concentration [mg/ml]: [mg of lyophilizate per ml of buffer solution]	2.5 mg/ml	Other remarks on the set-up of the enzyme reaction:	
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

Brief description of observed activity (under recommended conditions):	We only observed activity towards HYACARE and HYACARE 50 substrates using BCA assay under pH 7.0 conditions.
Detailed methodology of activity assay:	**BCA assay**: In a 2 ml Eppendorf we run the reaction using 190 µl of buffer solution, 2.5 mg/ml of Vibrio *sp.* lyophilizate or 20 µl supernatant of Vibrio *sp.* 1mg/ml of HYACARE or 2mg/ml of HYACARE. We leave the reaction for 72 hours at 37°C 950 rpm. After this time, we add 30 µl of BCA assay solution to a 384 well plate into each well. Then we add 10 µl of the sample to the appropriate well cover the plate and incubate at 70°C for 30 min: the colour formation will take place. Then we measure absorbance on spectrophotometer at 540 nm.
Verified positive and negative controls for the reaction:	BCA assay → Negative control: Only HA, only HA 50, only lyophilizate with buffer solution.

Tested range of conditions:			
Temperature [°C]:	37 °C	Substrate:	HYACARE (Evonic) HYACARE 50 (Evonic)
pH [-]:	150mM NaCl (pH 7), 300mM NaCl (pH 7), 0 mM NaCl (pH 4.0, 5.0,6.0, 7.0 and 8.0).	Substrate concentration [unit]:	HYACARE → 1mg/ml HYACARE 50 → 2mg/ml
Medium (water, buffer, salt, cofactors, etc.):	40mM HEPES buffer (150 mM, 300mM NaCl, pH 4.0, 5.0, 6.0, 7.0 and 8.0)	Expected product(s) and their quantities:	Reduced sugars
Time / duration:	72 hours	Analytical methods:	BCA assay
Enzyme concentration [mg/ml]:	2.5 mg/ml *supernatant was also used in the assay but there was no activity	Other remarks:	

Attachment (additional figures, descriptions, etc.)



Annex Fig. 41. BCA assay. *Vibrio alginolyticus* #23 lyophilizate activity with HA. Values of absorbance at 540 nm.

Enzyme specification sheet

Original version:	31.01.2024	CSIC	Paula Vidal	p.vidal.ramon@csic.es
Last update:	31.01.2024	CSIC	Paula vidal	p.vidal.ramon@csic.es

Enzyme:	**#14**	**FE_ID 9**

Enzyme class:	List of enyzme preparations (quantities for industrial tests have already been shared with partners):	Target application sector(s):
choose or enter	1. B01| CSIC | Ec_InCel_His_pur (Lot: 25.05.2022)	☒ Detergents ☐ Textiles (specify) ☐ Oil removal ☐ Dye removal ☐ Cosmetics ☐ Other:_____

Enzyme origin

Identifying partner:	CSIC	Type of expression:	Intracellular	Mode of application:	Solubilised enzyme
Original host:	*E. coli* BL21	Purification and formulation:	[formulation] [enzyme fraction]	Measured activity [U/g]:	5.43 U/g Activity measured with Glyceryl tributirate
Further characteristics / comments:		[text]			

Production batch: [B01]

Producing partner:	CSIC	Type of expression:	Intracellular	Mode of application:	Soluble enzyme
Production host:	*E. coli* BL21	Purification and formulation:	[formulation] [enyzme fraction]	Measured activity:	5.42 U/g
Further characteristics / comments:		[text]			

Application 1: Detergents

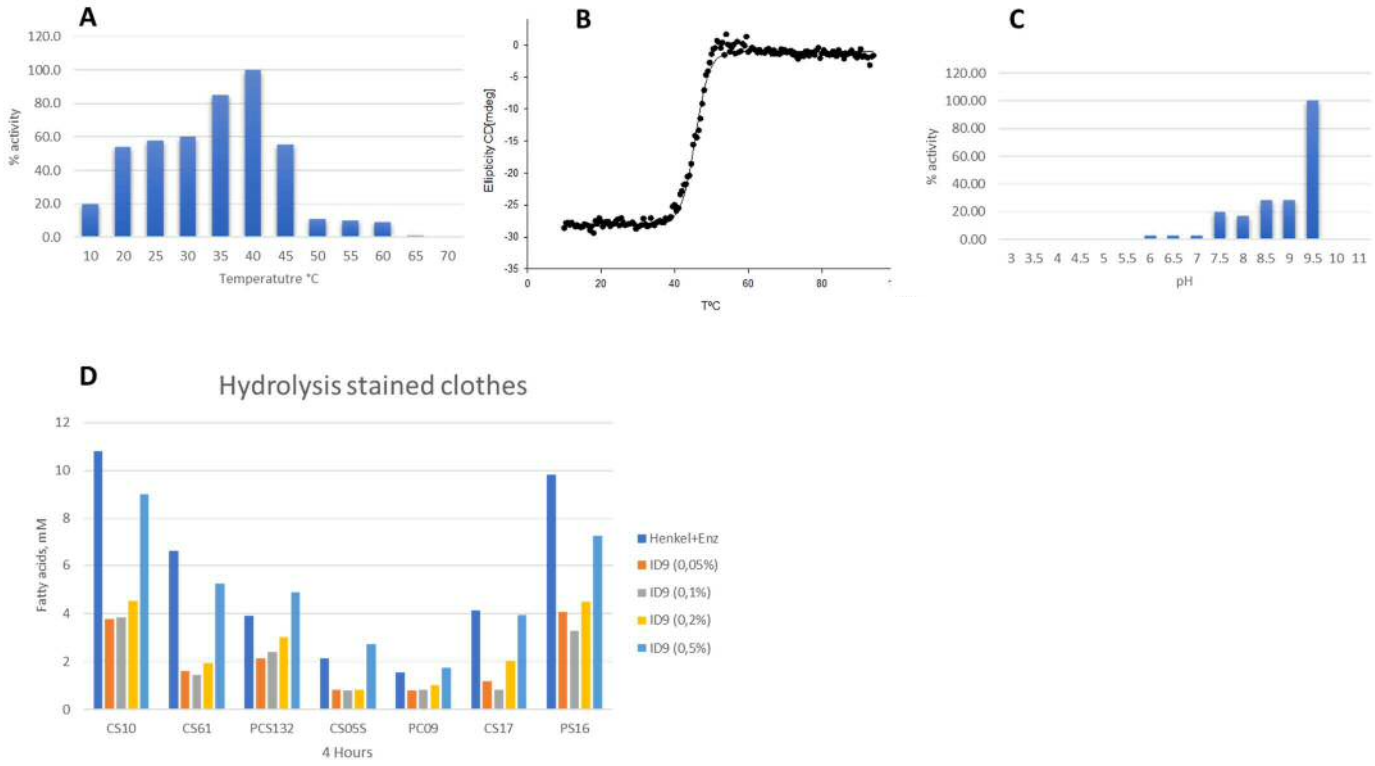
Recommended application conditions (from research perspective):			
Temperature [°C]*:	40 °C (See Annex Fig. 42A)	Substrate:	Stained clothes PC-09, C-S-05S, P-S-16, C-S-17, PC-S-132, C-S-61, C-S-10. Raw textile.
pH [-]*:	9.5 (optimal) 8.5-10.0 (acceptable) (Annex Fig. 42C)	Substrate concentration [g_c/L]: Grams of stained clothes per L of wash liquor	40 g_c/L
Medium (water, buffer, salt, cofactors, etc.)*:	Washing liquor (HENKEL®) (2.5 g/L)	Expected product(s) and their quantities:	Fat-free clothes (solid), glycerol (soluble), free fatty acids (soluble); exact composition unknown
Time / duration:	4 hours	Analytical methods:	Free fatty acids: Colorimetric method using the NEFA-Kit. Other methods not tested.
Enzyme concentration [mg/ml]: [mg of enzyme per ml of buffer solution]	3.87 mg/ml	Other remarks on the set-up of the enzyme reaction:	
** Optimum conditions and acceptable range/span for industrial application; if available provide graphs/data on the T and pH sensitivity/dependency of enzyme activity as attachment on last pages and cite respective figures here.*			

Brief description of observed activity (under recommended conditions):	For detergent, ID9 (B01) shows higher activity for some stained clothes compared to HENKEL® Liquid Laundry Detergent_A with enzymes like C-S-10 and PC-S-132 (Annex Fig. 42D).
Detailed methodology of activity assay:	Our small-scale assays and analytics are set-up as follows: 1) Using a circle hole puncher, a small piece (5 mm diameter, 4 mg) of each of the stained clothes are cut [stained clothes include 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); 2). The small piece is added to a 2-ml safe-lock Eppendorf® polypropylene tubes (ref. 0030 120.094, Greiner Bio-One GmbH, Kremsmünster, Austria); 3) Then, 100 µl of washing liquor are added; and 4) The appropriated amount of enzyme is added, and the reaction is maintained under the set-up experimental conditions, after which the release of fatty acids is determined by a colorimetric method using the NEFA-Kit (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany), as follows: in a 96 well plate, 10 µl of the reaction solution + 100 µl of NEFA solution 1 (R1a) are transferred. Following 6 min incubation at 37°C, 50 µl of NEFA solution 2 (R2a) are added. After 6 min incubation at 30°C, the samples' absorbance is measured at 550nm using a Synergy HT Multi Mode Microplate Reader (Agilent, Madrid, Spain).
Verified positive and negative controls for the reaction:	Positive control: HENKEL® Liquid Laundry Detergent_A including enzymes. Negative control: Washing liquor buffer (without enzymes)

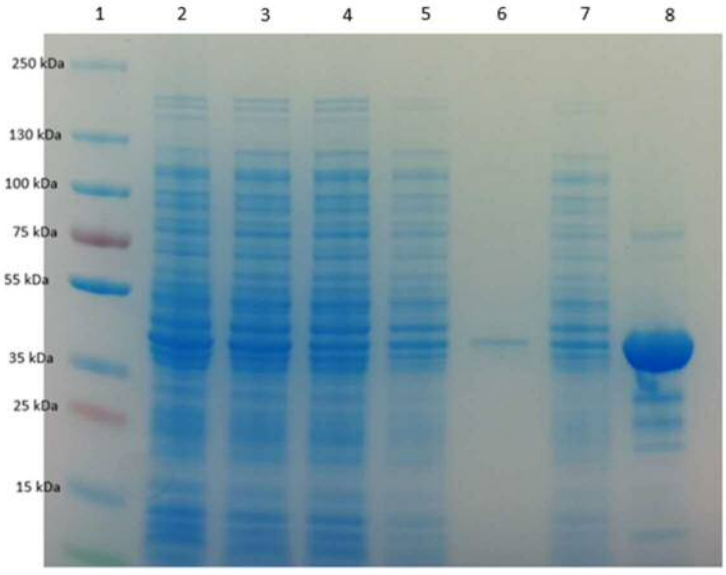
Tested range of conditions:

Temperature [°C]:	10-70 °C	Substrate:	Stained clothes PC-09, C-S-05S, P-S-16, C-S-17, PC-S-132, C-S-61, C-S-10. Raw textiles.
pH [-]:	3.0 - 11.0	Substrate concentration [g_C/L]:	40 g_C/L
Medium (water, buffer, salt, cofactors, etc.):	Washing liquor (HENKEL®) (2.5 g/L)	Expected product(s) and their quantities:	Fat-free clothes (solid), glycerol (soluble), free fatty acids (soluble); exact composition unknown
Time / duration:	0.3-4 h	Analytical methods:	Free fatty acids: Colorimetric method using the NEFA-Kit. No other method tested.
Enzyme concentration [mg/ml]:	0.8-4 mg/ml	Other remarks:	

Attachment (additional figures, descriptions, etc.)



Annex Fig. 42. Characteristics of ID9. **A**. Thermal profile of ID9 (B01); the data represents the relative percentages (%) of specific activity (U/mg) compared with the maximum activity using tripropionin (100mM) as substrate. **B.** Denaturation temperature of Lip9 as determined by circular dichroism. **C.** pH profile of ID9; the data represents the relative percentages (%) of specific activity (U/mg) compared with the maximum activity using pNPC3 as substrate. **D**. Concentration (mM) of free fatty acids released by ID9 (B01) from stained clothes under recommended application conditions.



Annex Fig. 43. SDS-PAGE Lipase Lipase_NODE_494_length_5773_cov_3.272419_27 (ID9). Lane 1. Molecular weight marker. Lane 2. Insoluble proteins. Lane 3. Soluble proteins. Lane 4. Flow through. Lane 5. 1st washing. Lane 6. 2nd washing. Lane 7. 3rd washing. Lane 8. Pure protein.

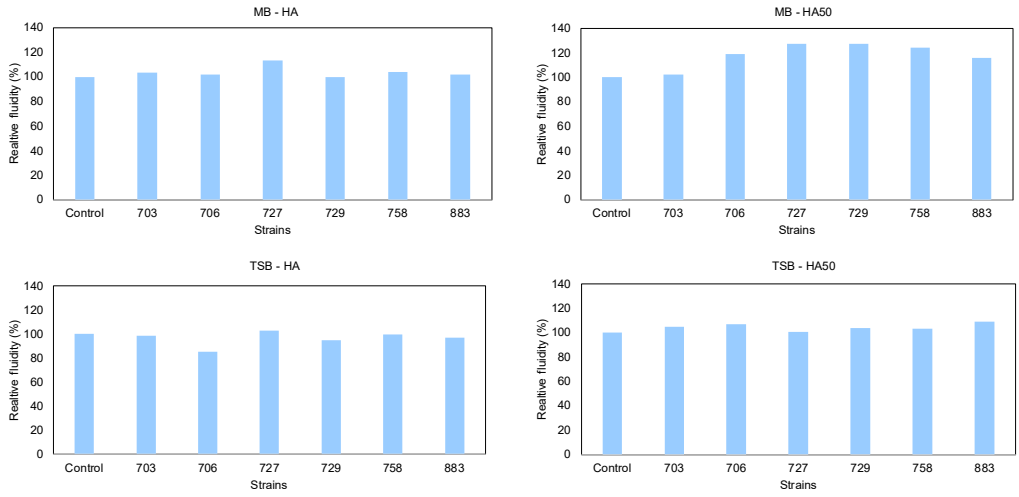
Supplementary Material

D6.2 Report on fermentation, DSP and activity verification for 18 Pre-Lead enzymes

Date: 31 Jan 2024

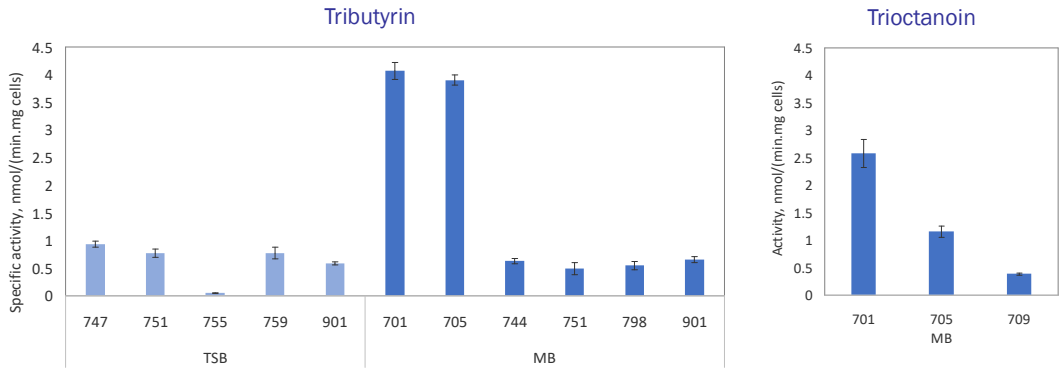
Fermentation conditions

The amount of enzyme produced per cell may vary significantly in response to the culture medium composition, as different metabolic pathways may be induced/activated. When comparing the effect of marine broth (MB) and tryptic soy broth (TSB) used for bacterial growth in the presence of hyaluronic acid, the highest activities were observed MB supplemented with Hyacare 50 (HA50) from Evonik (Supplementary Fig. 1).



Supplementary Fig. 1. Relative fluidity of hyaluronic acid solutions after treatment with hyaluronidases produced by different strains in MB and TSB. Bioconversion conditions: 100 µL of culture supernatant, 900 µL of 2 mg/mL of HA or HA50 in phosphate buffer; 24-well plate incubated at 30 °C and 100 rpm. Measurements shown were made after 48h of bioconversion.

Similarly, enzyme activity towards tributyin and trioctanoin could be increased by growing the native strains in MB or TSB (Supplementary Fig. 2). The supplementation of the culture medium with olive oil could also increase lipase activity in some strains (data not shown).



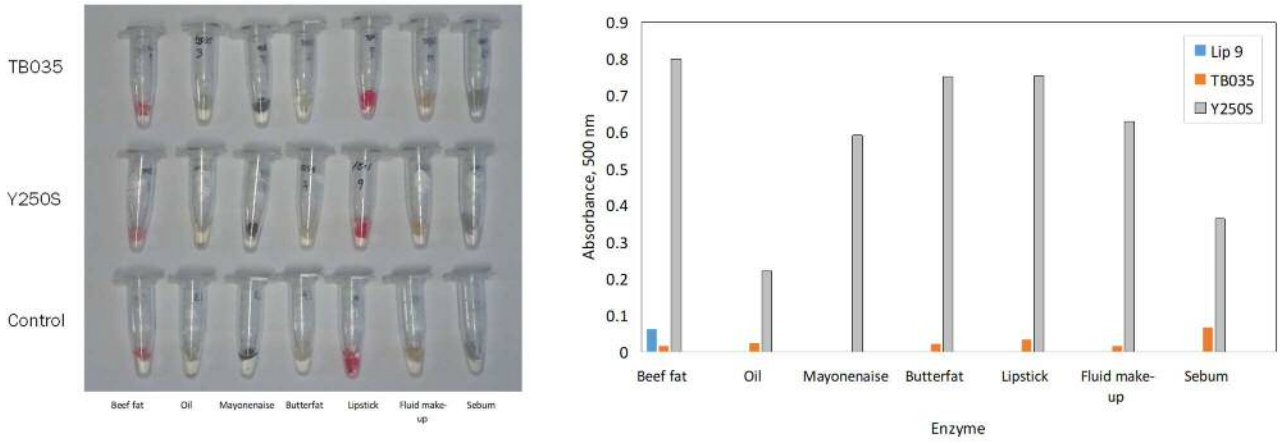
Supplementary Fig. 2. Effect of culture medium used for cell growth on lipase activity. Bioconversion conditions: 5 mM substrate and 0.14 mM phenol red in EPPS buffer, and 9.09 µl of the sample. Total volume: 100 µL; 96-wells microplate.

Activity verification for pre-lead enzymes

The enzymes received from FuturEnzyme partners were tested under conditions mimicking real industrial conditions in small scale bioreactors.

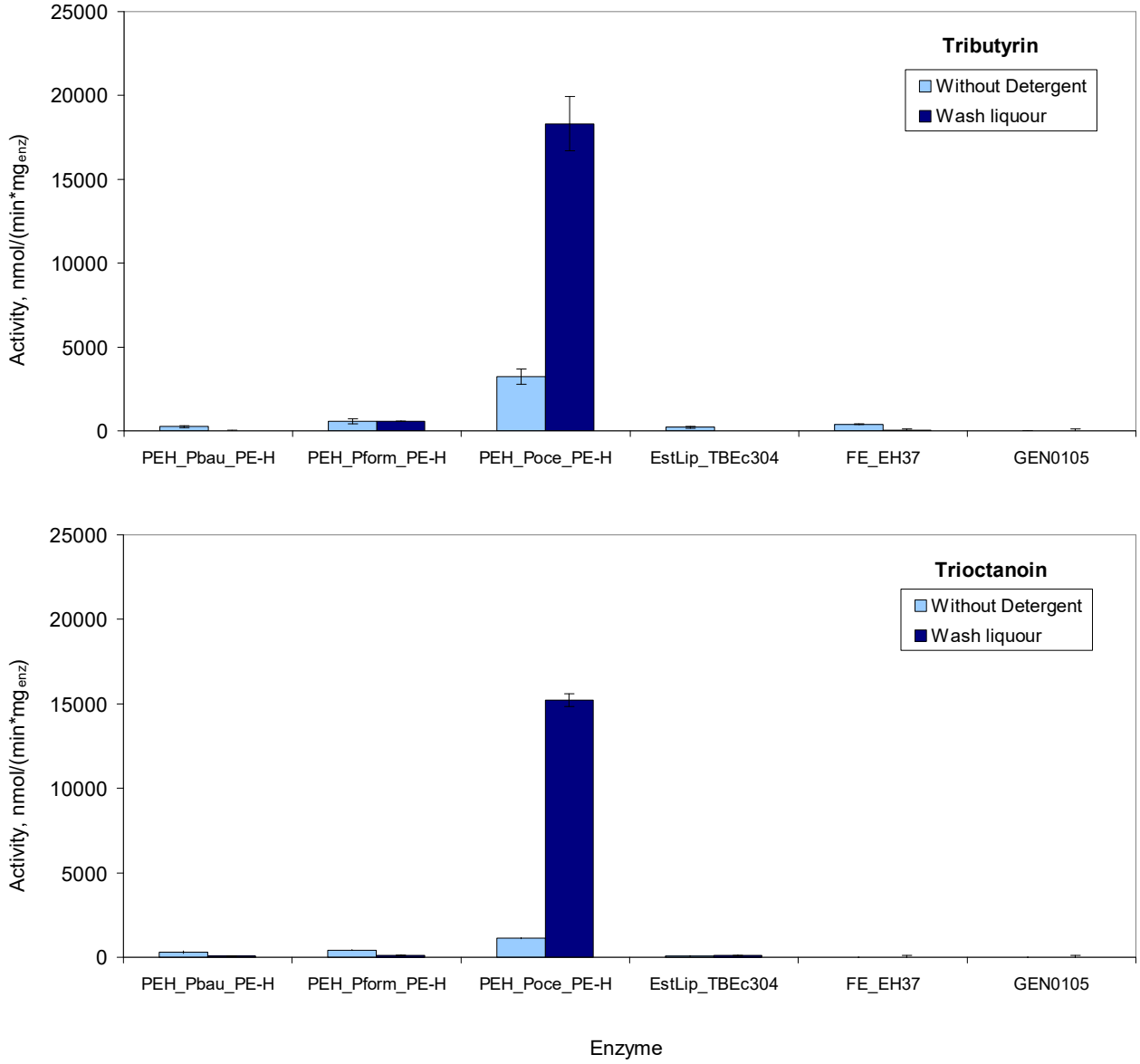
Detergents - Lipases

The enzymes Lip 9, TB035 and Y250S produced in large scale by BioSynth were tested in standard soiled textiles (Supplementary Fig. 3). Using a NEFA-HR(2) kit to monitor the removal of the stains, the results showed that Y250S was the most efficient enzyme, being more active with beef fat, butterfat and lipstick, when compared to the other tested enzymes, under the tested conditions.



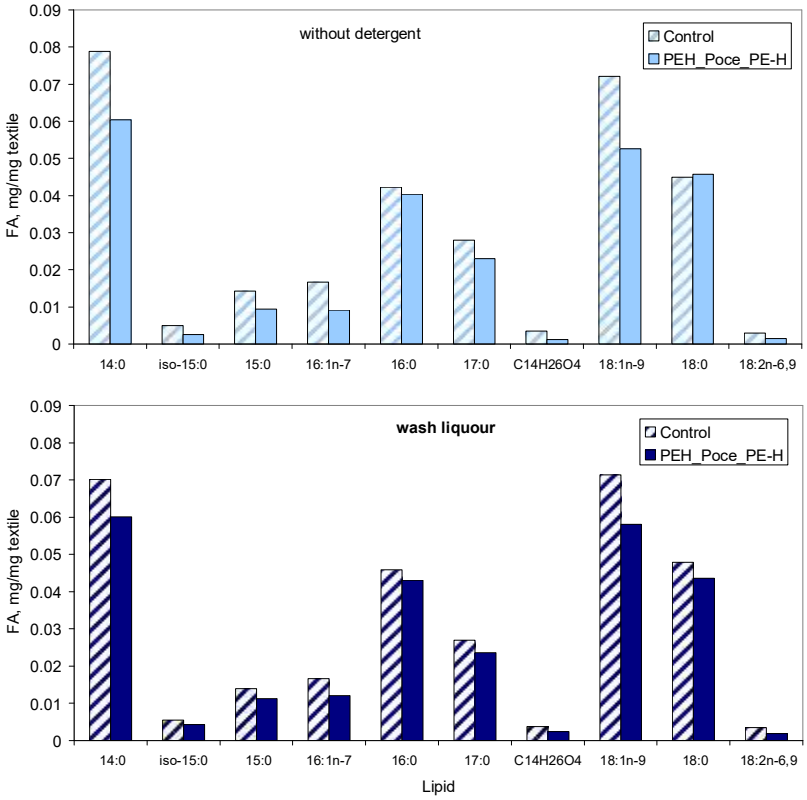
Supplementary Fig. 3. Removal os stains from soiled textiles by Lip 9, TB035, and Y250S enzymes. Reaction conditions: 200 µL Tris-HCl 40 mM pH 8 buffer; 10 µL enzyme solution at 1.5 mg/mL; disks of soiled textiles previously weighted. Reactions carried out for 24h at 30°C and 200 rpm in 1.5 mL eppendorfs. Analysis carried out using a NEFA-HR(2) kit.

The lyophilized enzymes PEH_Pbau_PE-H, PEH_Pform_PE-H, PEH_Poce_PE-H, EstLip_TBEc304, FE_EH37, and GEN0105, produced at large scale by BioSynth, were tested for their activity and stability in the presence of wash liquour. This is a solution containing detergent supplied by Henkel, at the suggested concentrations to mimic a washing cycle. The enzyme PEH_Poce_PE-H presented the highest activity in the presence of wash liquour, being able to convert both tributyrin and trioctanoin at much faster rate in the presence of wash liquour than in buffer (Supplementary Fig. 4).



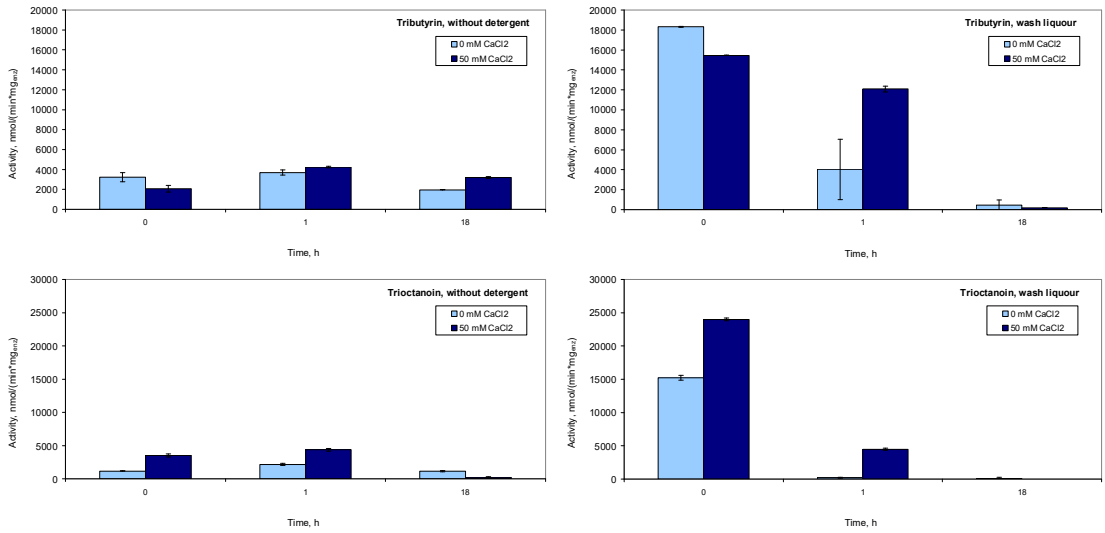
Supplementary Fig. 4. Effect of wash liquour on enzyme activity. Reaction conditions: 90.9 µL EPPS 2.5 mM pH 8 buffer; 9.09 µL enzyme solution at 0.5-5 mg/mL; substrate 100 mM in DMSO; 90.9 µL phenol red 0.304 mM in EPPS buffer; with/without 3.1 g/L detergent. Assays carried out at 30°C and 200 rpm.

The enzyme PEH_Poce_PE-H was also able to degrade both saturated and unsaturated fatty acids in textiles, even in the presence of wash liquour (Supplementary Fig. 5). The largest changes were observed with the saturated myristic acid (14:0) and unsaturated 9-octadecenoic acid (18:1n-9).



Supplementary Fig. 5. Fatty acid profile of soiled textiles before and after cleaning with PEH_Poce_PE-H, in systems with and without wash liquour. Reaction conditions: 4.9 mL Tris-HCl 40 mM pH 8; 100 µL enzyme at 3.75 g/L; 1 cm^2 of beef fat soiled cloth; 40°C and 800 rpm; in glass vials. Analysis by GC-MS after 24h of biotransformation.

Since Calcium ions may influence the performance of enzymes, their effect on PEH_Poce_PE-H was assessed during the conversion of tributyrin and trioctanoin at a concentration of 50 Mm $CaCl_2$ (Supplementary Fig. 6). Although a significant decrease in activity was observed without detergent and $CaCl_2$, the stability was increased in the presence of the latter. In fact, the activity in the presence of wash liquour was larger than in the system without detergent, and the stability could also be improved in the first hour, which should be enough for a washing cycle.

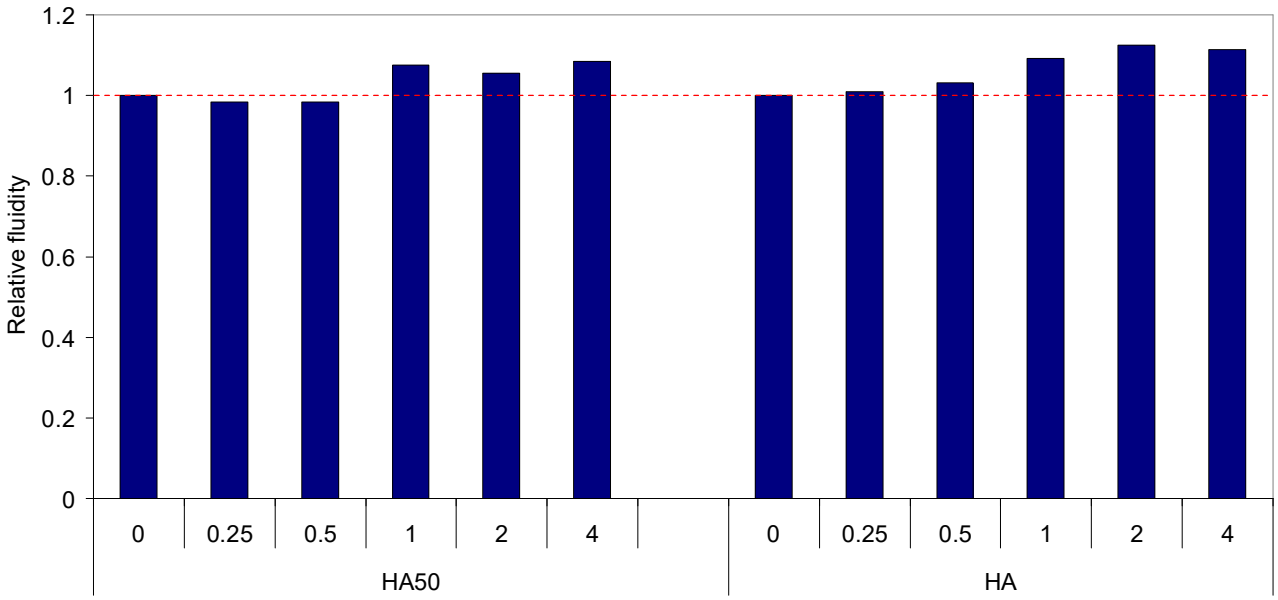


Supplementary Fig. 6. Effect of calcium on the activity and stability of PEH_Poce_PE-H. Reaction conditions: 90.9 µL EPPS 2.5 mM pH 8 buffer; 9.09 µL enzyme solution at 0.5 mg/mL; substrate 100 mM in DMSO; 90.9 µL phenol red 0.304 mM in EPPS buffer; with/without 3.1 g/L detergent. 96-well plates at 30°C and 200 rpm.

Cosmetics - Hyaluronidases

The enzyme sent by BioC-CheM Solutions, produced by *Vibrio alginolyticus* #23 in large scale, did not show activity at 30°C and 100 rpm, using 2 mg/mL Hyacare (HA) and HA50 at different enzyme concentrations. Other conditions are currently being tested.

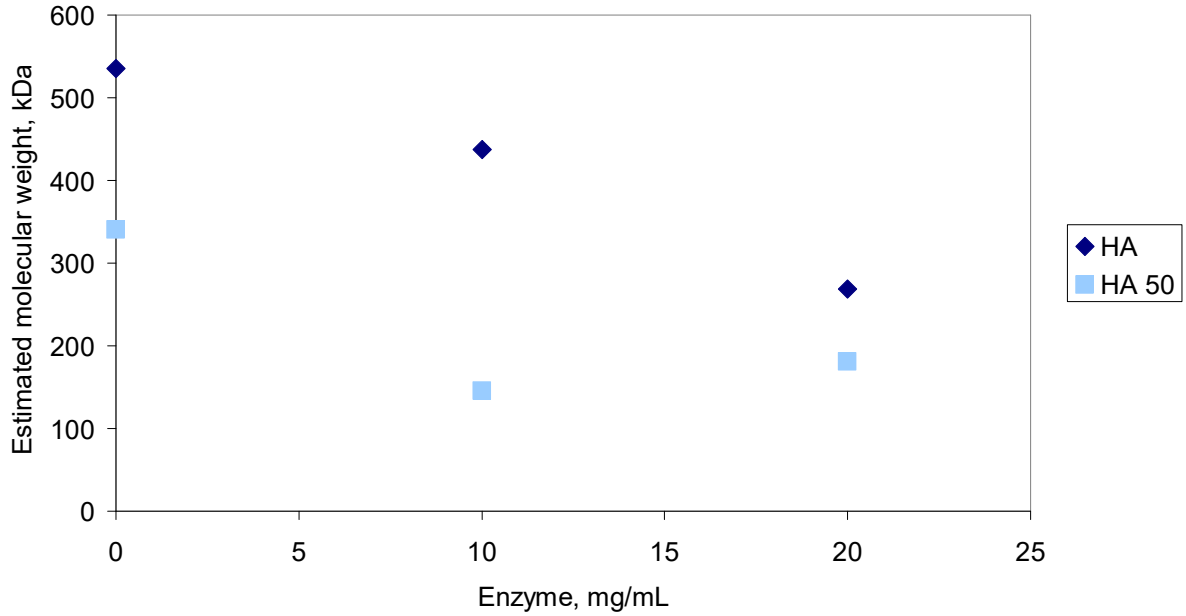
The enzyme Hyal_HRDSV_2334, produced in large scale by Biosynth, presented increased activity with increasing concentrations of both HA and HA50 (Supplementary Fig. 7).



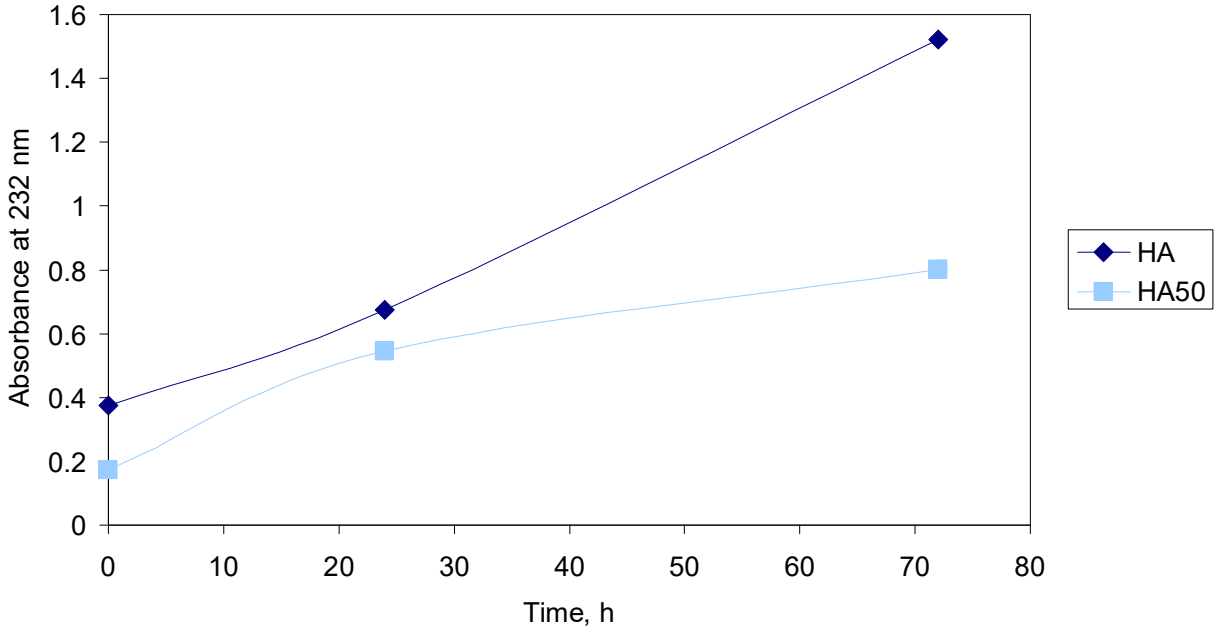
Supplementary Fig. 7. Relative fluidity of HA and HA50 solutions treated with the enzyme Hyal_HRDSV_2334. Reaction conditions: 2 mg/mL HA or HA50; 0-4 g/L of enzyme; 30°C and 100 rpm in 24-well microtitre plates. Reaction monitored every 24h for 4 days; results after 24h are shown.

The estimated molecular weight of the degraded HA and HA50 decreased with increasing amount of enzyme, indicating that the enzyme was apparently able to decrease the size of hyaluronic acid (Supplementary Fig. 8). This test was made by measuring the migration of the solution containing hyaluronic acid on a strip of paper as a proxy of viscosity. When the enzymes are active, they cut the hyaluronic acid molecule and the solution becomes more fluid, travelling a larger distance. A calibration curve relating the amount of wetted paper and the molecular size of hyaluronic acid allowed the estimation of the size of the resulting molecule during the enzymatic assays.

The presence of digested hyaluronic acid products such as those formed by the action of hyaluronidase lyase could also be found by measuring the absorbance at 232 nm (Supplementary Fig. 9).



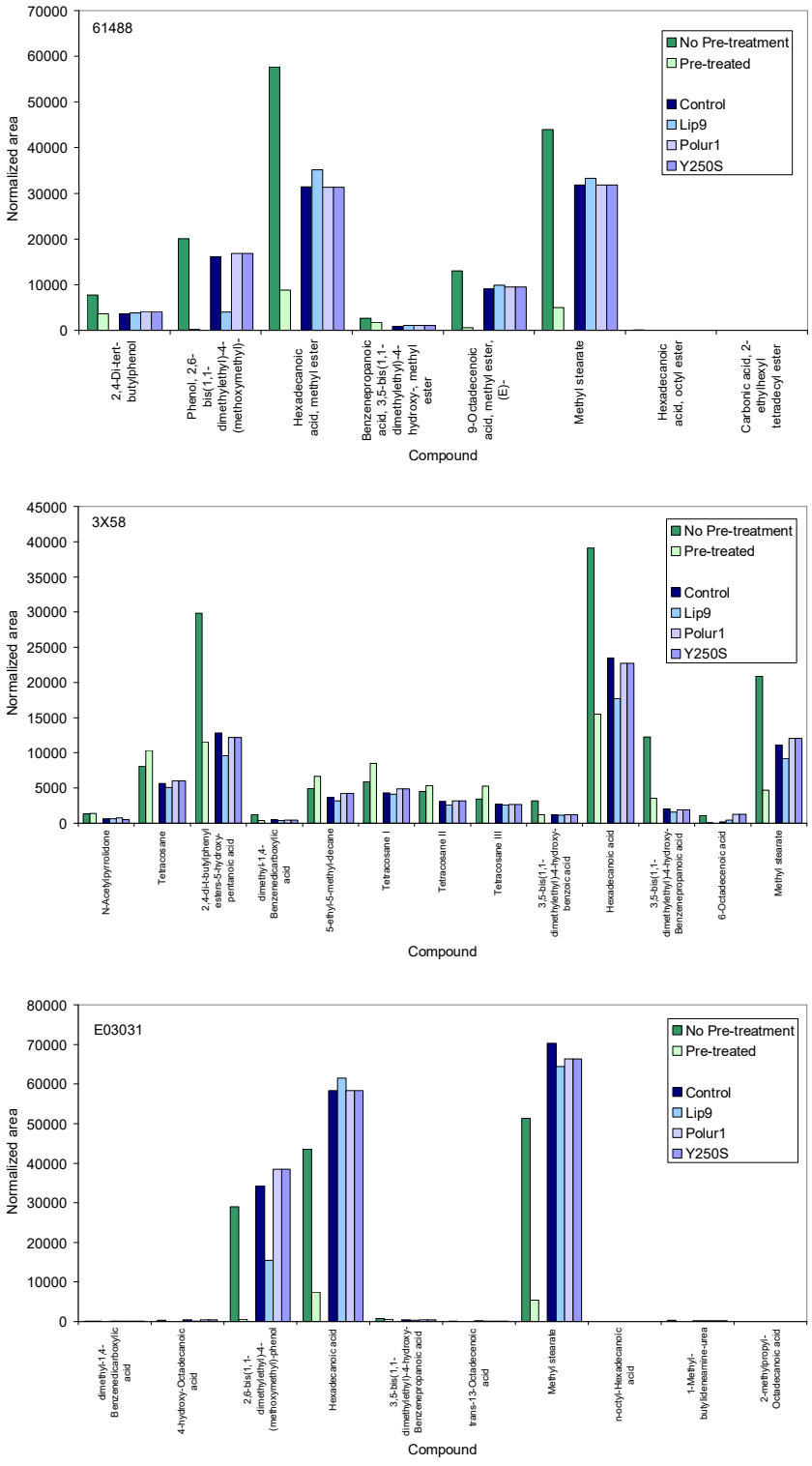
Supplementary Fig. 8. Effect of enzyme concentration on the estimated molecular weight of the resulting hyaluronic acid after degradation of HA and HA50 in 24h



Supplementary Fig. 9. The presence of digested hyaluronic acid products such as those formed by the action of hyaluronidase lyase.

Textiles – lipases

The enzymes Lip9, Polur 1 and Y250S were used by Schoeller to clean the fabrics of the spinning oils. In general, the enzymes decreased the amount of several compounds present in sample 61488, when compared to the no-treatment control (Supplementary Fig. 10). However, they did not perform as well as the current treatment used (pre-treated samples). In the fabric 3X58, the enzymes were able to degrade some compounds at nearly the same extent as the chemical treatment or even better. However, they were not able to remove the spinning oils from the fabric E03031.



Supplementary Fig. 10. Enzyme treatment of 61488, 3X48 and E03031 fabrics made at Schoeller and analysed at IST-ID by GC-MS.