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REPORT ON SMALL/MEDIUM VALIDATION TRIALS OF 18 BEST PRE-SELECTED ENZYMES

DELIVERABLE NUMBER D7.1

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Document information sheet

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Summary

1. Scope of Deliverable

We committed to delivering a report detailing the successful integration of project enzymes (selected from at least 18 pre-selected enzymes) as ingredients or components of production processes, resulting in improved properties of liquid detergents and/or unit dose caps products, textiles, and cosmetic products. This report provides comprehensive information on the results obtained from washing trials, detergent stability, conditions for textile pretreatment and finishing, textile characteristics, conditions for the production of hyaluronic acid hydrolytic products, and formulation conditions and characteristics of cosmetic products. The data presented herein stem from extensive testing conducted by both academic and industrial partners, demonstrating the successful achievement of the deliverable.

2. Introduction

Work Package 7 (WP7) aims to upscale appropriately dimensioned trials for integrating the best enzymes into leading premium liquid detergents, producing textiles with diverse requirements, and creating defined size HA products for integration into cosmetic products. The objective is to enhance existing products worldwide, making them more environmentally friendly, innovative, and functional. Enzymes will be utilized to achieve sustainability goals in detergent formulations, improve textile dyeability and washability, and facilitate the production of cosmeceutical ingredients. The success of WP7 hinges upon Deliverable D7.1, which is the culmination of the activities outlined below. These tasks collectively contribute to the development and optimization of enzyme-based solutions for detergent, textile, and cosmetic applications, aligning with the overarching goals set forth within the project's scope. Additionally, the development of expression and production systems for the enzymes utilized in WP7 has been instrumental in facilitating these activities. Detailed descriptions of these systems are provided in Deliverable D6.1 and D6.2 (Jan 2024).

1. Task 7.1. Lead Partner: HENKEL. Participants: CSIC, IST-ID, Bio_Ch, CLIB.
   Summary: In Task 7.1, the aim is to identify new enzymes for detergent applications through small to medium-scale trials. These trials will assess the performance of various enzyme formulations obtained under different washing conditions. CSIC, IST-ID, and Bio_Ch will conduct tests using consumer-relevant stains and soiled fabrics provided by HENKEL. The trials will focus on optimizing cleaning results while reducing the use of chemical active ingredients. The best-performing enzymes will be selected for upscaling trials and detergent formulations, which will be evaluated in full-scale wash trials by HENKEL.
2. Task 7.2. Lead Partner: SCHOELLER. Participants: CSIC, IST-ID, Bio_Ch, CLIB
   Summary: Task 7.2 aims to identify new enzymes for textile applications through small to medium-scale trials. These trials will assess the performance of enzyme formulations in textile cleaning. CSIC and IST-ID will conduct initial tests, followed by upscaling trials by SCHOELLER.
3. Task 7.3. Lead Partner: EVO. Participants: CSIC, IST-ID, Bio_Ch, CLIB
   Summary: In Task 7.3, the goal is to identify hyaluronidases for cosmetic applications through small to medium-scale trials. These trials will assess the performance of enzyme formulations obtained in Task 6.1 in HA hydrolysis. CSIC, IST-ID, and Bio_Ch will conduct tests to determine optimal conditions for HA hydrolysis. The selected enzymes will be further used for upscaling trials to produce HA hydrolysis products of defined size. These products will be integrated into existing commercial EVO Hyacare® products, and their compatibility and stability will be tested.

Based on all of the above, this document summarizes:

1. The preselected enzymes produced in *Escherichia coli* and *Pichia pastoris* at a gram scale for validation, including the quantities produced and supplied to academic and industrial partners.
2. Methodological details and validation results obtained by academic partners on a small scale.
3. Methodological details and validation results obtained by industrial partners on a medium to large scale.
4. Next steps based on validation results and selection of best enzymes for future actions.

3. Methodology: Materials and Methods

3.1. Materials and Methods for Production in E. coli, P. pastoris and Native Hosts (+12 L scale)

Fermentation at gram scale of selected enzyme candidates (see Table S1) was performed as detailed in Deliverables D6.1 (Best 18 Pre-Lead Enzyme Materials Obtained at Gram Scale for Real-Life Tests) and D6.2

(Report on Fermentation, DSP and Activity Verification for 18 Prelead Enzymes) submitted on Jan 2024. Briefly, the following productions systems were set up: (1) *E. coli* BL21(DE3) or *E. coli* LOBSTR and the vectors pET-45b(+) or pVec11, supporting His_6-tag isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced intracellular expression, and Ni-NTA His-Bind resin purification (Fig. 1); (2) *Pichia pastoris* AOX locus and the Biosynth-owned plasmid pPichia57, supporting methanol-induced extracellular expression (Fig. 2); (3) native host such as *Vibrio alginolyticus* strain IAMC-CNR#23 and *Coriolopsis trogii* (Fig. 3). For more details, see D6.1 and D6.2 (Jan 2024).

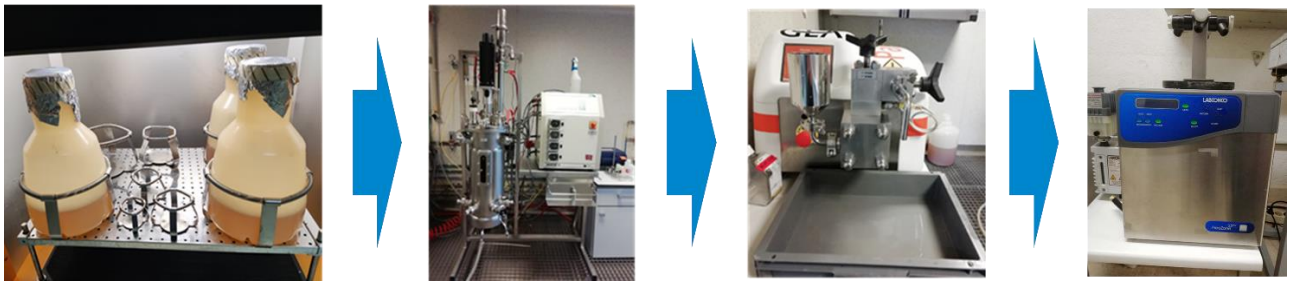

Fig. 1. CSIC Fermentation Development and Production Pipeline of selected enzymes at gram scale in *Escherichia coli*: The lead candidates were produced using *E. coli* as the host organism. Screening and small-scale production were conducted using a set of orbital shakers capable of accommodating flasks ranging from 50 to 2000 mL. Fermentation was carried out in a Fermenter Biostat C Plus (B. Braun Biotech), followed by downstream processing using a French Press (GEA Niro Soavi). Lyophilization was employed for long-term storage of the produced enzymes, utilizing a Labconco Freeze Dryer Model Free Zone 2.5.

Fig. 2. BioSynth Fermentation Development and Production Pipeline of selected enzymes at gram scale in *Pichia pastoris*: The lead candidates were expressed in *P. Pastoris* as host organism. Fermentation development commenced with a 4x 1L scale multi-parallel fermenter, after which the optimal process for each candidate was transferred to a 10 L pilot scale. The target enzymes were subsequently isolated from the culture supernatant, transferred into the target buffer using ultrafiltration, and finally lyophilized for storage.

Fig. 3. Bio_Ch Fermentation Development and Production Pipeline of selected enzymes at gram scale in native hosts: The lead candidates were produced using native hosts. Eppendorf shakers were utilized for screening the best media, temperature, shear

stress, and oxygen requirements by the isolated non-recombinant strains. A set of three orbital shakers could accommodate up to 150 500-mL flasks for screening and small-scale production purposes. Fermentation was carried out either in a 20 L lab-fermenter from B-Braun or a 30 L lab-fermenter from Bioengineering. The fermentation parameters were automatically controlled and continuously monitored. Lyophilization was employed for long-term storage of the produced enzymes. Lyophilization was performed in 25-mL lyovials to produce "ready-to-use and standard samples," which could be conveniently stored at 4°C or -20°C. The lyophilizer allowed processing of up to 450 vials per cycle.

3.2. Analytics for Validations at Laboratory Scale: Academic Partners

Validation tests were performed using appropriate spectrophotometers and analytical techniques detailed in Deliverable D3.2 (Standard assays, analytics and calculations for monitoring enzymatic performance). Below, a summary of the methods in brief.

Experimental Setup for Evaluating Washing Efficiency of Stained Clothes at Laboratory Scale. 30 °C/40 °C; pH 8; Tris-HCl buffer at 40 mM; Stains tested (Fig. 4): 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 (about 4 mg) using a circle hole puncher; 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, as a control. Tubes were incubated for 30 min to 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 (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany), according to standard procedure: 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). All reactions and analyses were performed in triplicate (n = 3).

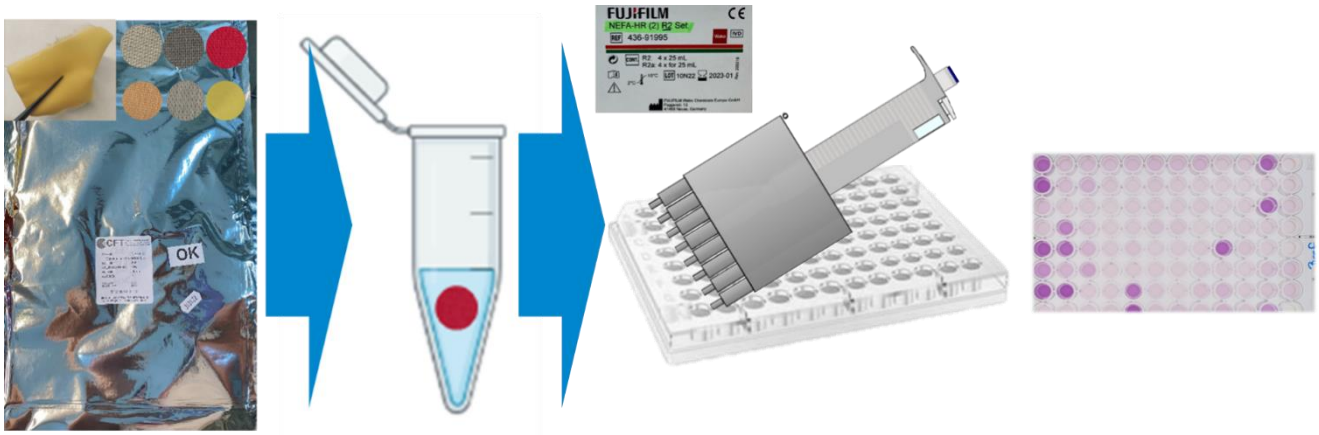

Fig. 4. Experimental Setup for Evaluating Washing Efficiency of Stained Clothes PC-09, C-S-05S, P-S-16, C-S-17, PC-S-132, C-S-61, and C-S-10 at Laboratory Scale Using the NEFA-HR(2) Kit.

Experimental Setup for the degradation of hyaluronic acid at laboratory scale. The enzymatic reaction mixture consisted of 100 µL of the hyaluronidase (HAase) supplied by BioSynth (Hyal_HRDSV_2334) and a 2 mg/mL HA (Fig.1) solution in H_2O. After incubation at 35 °C for an appropriate time, the reaction mixture was inactivated by incubating the samples in a Thermomixer (Eppendorf, Hamburg, Germany) for 5 min at 95 °C. Time course samples were analyzed by DNS colorimetric assay and in order to see in which time we had to stop the reaction to obtain the desired molecular weight range Hyalo oligos (≤ 5kDa), samples were diluted with water (1:10) and then analyzed using high performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) on an ICS3000 Dionex system (Dionex, Thermo Fischer Scientific Inc., Waltham, MA, USA) consisting of a SP gradient pump, an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode, an auto-sampler (model AS-HV). All eluents were degassed by flushing with helium. A pellicular anion-exchange 4 x 250 mm Carbo-Pack PA-100 column

(Dionex) connected to a 4 x 50 CarboPac PA-100 guard column were used at 30 °C. Eluent preparation was performed with Milli-Q water, 50% (w/v) NaOH and sodium acetate trihydrate. The compounds were eluted by a gradient method in which the initial mobile phase was 30 mM NaOH and 270 mM of sodium acetate trihydrate for 45 min, then from minute 45 to minute 70, the mobile phase was increased gradually until it reached a concentration of 100 mM NaOH and 900 mM of sodium acetate, which was further maintained during 5 min. Finally, initial conditions were reset and maintained for 10 min to equilibrate the column, using a flow rate of 0.5 mL/min for. The peaks were analyzed using Chromeleon software (Dionex, Thermo Fischer Scientific Inc., Waltham, MA, USA). All reactions and analyses were performed in triplicate (n = 3).

3.3. Analytics for Validations at Laboratory Scale Using Real Substrates: Industrial Partners

Small scale validations were performed by industrial partners, using a series of experimental set ups briefly detailed below.

Experimental Setup for small scale wash performance and Mini Wash Trials. The lyophilised enzyme samples provided by BioSynth were dissolved in 20% PG in the same protein-concentration as a technical benchmark (IP3461). First, stability in detergent liquor was evaluated quantifying the residual activity in washing liquor (IP3461) @40°C, +/- Protease. Then, washing efficiency was evaluated in European detergent w/o enzymes, 6-fold stain (CS46B), 40°C, 1h, 600 rpm. Concentration row of lipase samples, pH checked before and after the measurement. More in details, the evaluation of the new lipase candidates involves assessing their stain removal performance in small-scale wash trials (Fig. 5). These trials are designed to simulate real-life, full-scale wash conditions experienced in consumers' homes. The enzymes are dissolved in 1 mL wash liquor of a real liquid laundry detergent product (which does not contain other enzymes) and wash performance is tested microtiter plate format (48 wells, each stain 1 cm in diameter). Different consumer relevant stains, containing fatty/oily soils are applied and evaluated in multiple replication. These test stains are commercially available from external providers like WFT-Testgewebe GmbH or CFT (Center for Testmaterials) The microtiter plates with the wash liquor, containing the new enzymes, are incubated at different temperatures while shaking, to simulate mechanical stress like in a real wash process. Temperature and incubation time are aligned to real consumer conditions (i.e. 60 min main wash cycle in a European front loader washing machine, cotton program). The decolorization/brightening of each stain is measured by a Mach5+ colour instrument (multi spectral camera) and is taken as a measure for stain removal performance. High loss of color = bright textile = good stain removal performance.

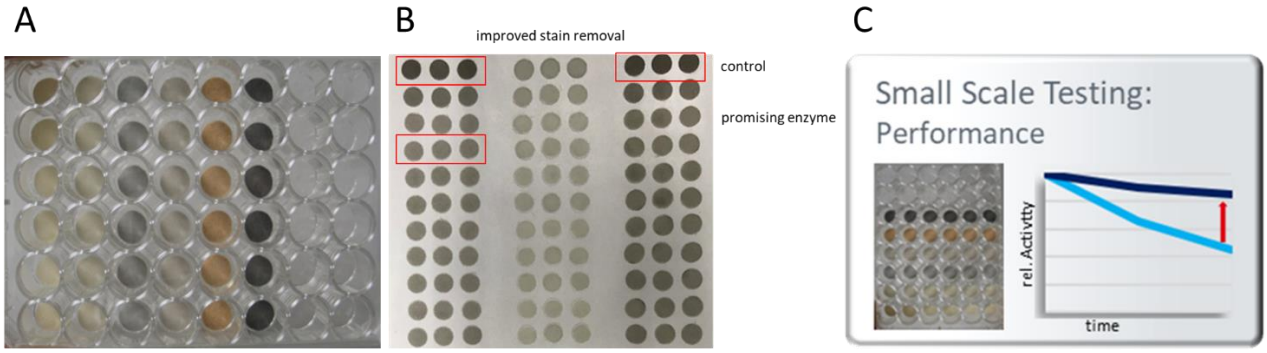

Fig. 5. Experimental Setup for Assessing Stain Removal Performance in Small-Scale Wash Trials to Simulate Real-Life, Full-Scale Wash Conditions at Consumers' Homes. A) Enzymes are dissolved in 1 mL of wash liquor from a genuine liquid laundry detergent product (which does not contain other enzymes). The wash performance is then tested in a microtiter plate format (48 wells, each stain measuring 1 cm in diameter), which contains various stains relevant to consumers. B) The microtiter plates, containing the wash liquor with the new enzymes, are incubated at temperatures and for durations aligned with real consumer conditions. C) The decolorization/brightening of each stain is measured using a Mach5+ color instrument (multi-spectral camera), which serves as a metric for stain removal performance.

Experimental Setup for external substrates (silicons, spinning oils, etc.) removal from the raw fabric samples. 1) Prepare 100 mL liquor at 30ºC; 2) Determine pickup; 3) Pad 4 items; 5) Wrap in foil and store for 24 hours; 6) Wash out at 60ºC without tools; 7) Drying at 150ºC; 8) Quantitative (A) and qualitative (B, C) analysis. A. Petroleum ether soluble substances - quantitative determination of the quantity: To determine the amount of material added, a defined amount of material is extracted in boiling petroleum ether and the amount in% is determined in relation to the weight of the material. B. Qualitative determination: In order to determine the qualitative composition of the application, the extract obtained is measured in the IR (infrared) spectrometer. Conclusions about the chemical basis of the extract can be drawn from the position of the absorption bands in relation to one another. C. Hot water - quantitative determination of the quantity: A defined amount of material is boiled in osmosis water for one hour. After the water has evaporated, the difference in weight of the extraction glass before and after the extraction is determined. Then the edition is determined in% in relation to the weight of the material. Experimental setup and equipment are briefly summarized in Fig. 6 and Fig. 7.

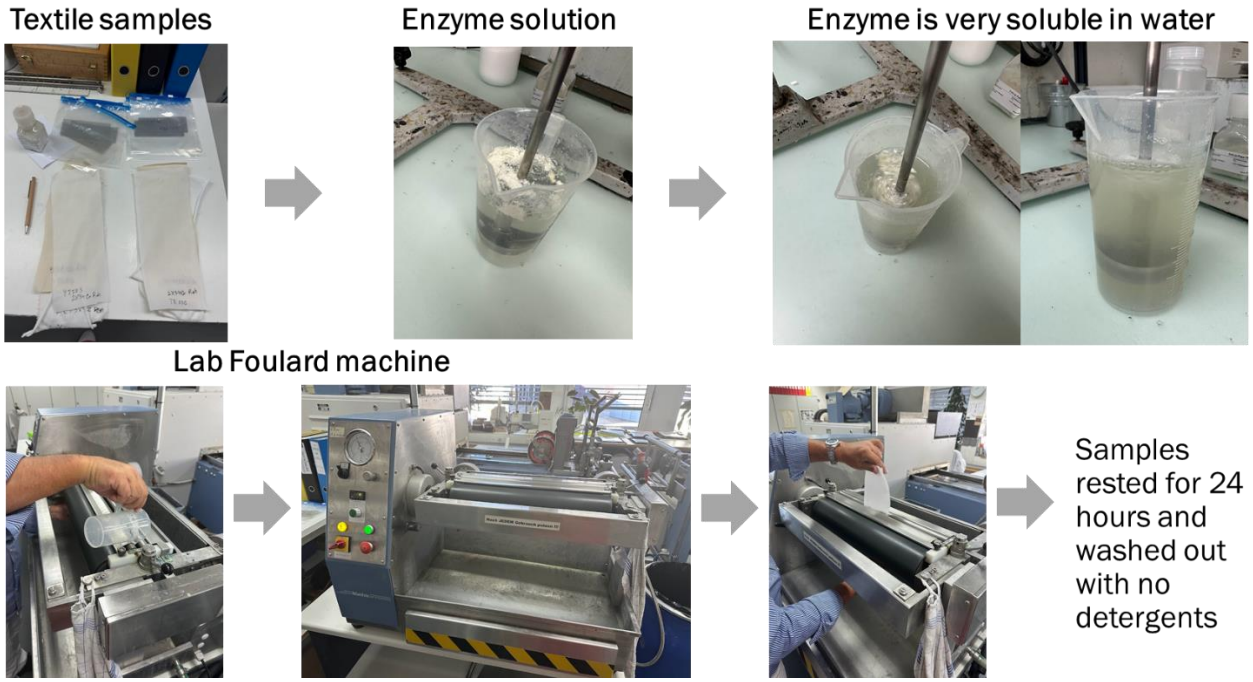

Fig. 6. Experimental Setup for Evaluating External Substrates (Silicons, Spinning Oils, etc.) Removal from the Raw Fabric Samples. A video showing the cleaning process is shown in the project intranet (see details in Section 10, Video S1).

Fig. 7. Equipment and Materials Evaluating External Substrates (Silicons, Spinning Oils, etc.) Removal from the Raw Fabric Samples.

Experimental Setup for the degradation of hyaluronic acid. Hyaluronic acid degradation is performed based on the method developed by Rapport et al., 1951. Steps: 1) prepare potassium buffer phosphate (KP) 150 mM pH 7; 2) add 0.0125 g/L BSA to the KP; 3) add 4 g/L Hyaluronic Acid (variable depending on the HA used, maximum value for Hyacare reported) to the KP+BSA solution; 4) incubate at 37°C under stirring, keep the pH at 7 by adding KOH 10M as required, and monitor the absorbance spectrophotometrically as OD600; 5) when the OD600 is stable (up to 24 hours required), add Hyaluronidase lyase (HL) (amount variable depending on activity and type of the enzyme); 6) incubate at 37°C with stirring, keep the pH at 7 by adding KOH 10M as required; 7) monitor the degradation according to Rapport et al., 1951; 8) stop the reaction by acidifying at pH 3 the solution with HCl; 9) store at 4°C or -20°C.

4. Enzymes Produced for Validation Purposes

To date, a total of 46 enzymes are in the production phase, utilizing *E. coli* or *P. pastoris* expression systems or fermentation of native hosts. Table S1 provides detailed information on these enzymes, summarizing those that have been produced, transferred, and validated by the partners in Table 1.

Table 1. Enzymes Already Produced at Large for Validation Purposes.

Enzyme	**Application**[1]			**Quantity of produced enzyme, expression host and partners**
	D	**T**	**C**	
FE_Lip9	Y	Y (O)	N	CSIC: 1.05 g (*E. coli*); BIOSYNTH: 55,7 g (*P. pastoris*)
FE_Lip9_mut (Val161Ser)	Y	Y	N	BIOSYNTH: 62.38 g (*P. pastoris*)
FE_ID 9	Y	N	N	CSIC: 2.0 g (*E. coli*)
FE_polur1	Y	Y (O)	N	BIOSYNTH: 28.36 g (*P. pastoris*)
EstLip_Dim_#008	Y	Y (O)	N	BIOSYNTH: 41.2 g (*P. pastoris*)
EstLip_Paes_TB035	Y	Y (O)	N	BIOSYNTH: 176 g (*P. pastoris*)
EstLip_PtEst1	Y	Y (O)	N	BIOSYNTH: 39.88 g (*P. pastoris*)
EstLip_TBEc304	Y	Y (O)	N	BIOSYNTH: 930 g (*P. pastoris*)
Paes_PE-H_Y250S	Y	Y (O)	N	BIOSYNTH: 94.95 g (*P. pastoris*)
PEH_Pbau_PE-H	Y	Y (O)	N	BIOSYNTH: 125 g (*P. pastoris*)
Pform_PE-H	Y	Y (O)	N	BIOSYNTH: 132 g (*P. pastoris*)
PEH_Poce_PE-H	Y	Y (O)	N	BIOSYNTH: 76.8 g (*P. pastoris*)
GEN0105	Y	N	N	BIOSYNTH: 32.7 g (*P. pastoris*)
GEN0095	Y	Y	N	BIOSYNTH: 33.4 g (*P. pastoris*)
Hyal_HRDSV_2334	N	N	Y	BIOSYNTH: 79.4 g (*P. pastoris*)
VA-23_PL8B	N	N	Y	BIO_CH: 1.5 grams crude extract (*V. alginolyticus*)
FE_EH37	Y	Y (O)	N	BIOSYNTH: 51.2 g (*P. pastoris*)
Lac3379-1	N	Y (D)	N	BIO_CH: 10 g crude extract, approx 1 g p (*Coriolopsis trogii*)
pVec11	N	Y (D)	N	BANGOR: few milligrams scale (*E. coli*)

[1]Abbreviations: D, Detergents; T, Textiles; C, Cosmetics; O, Oil removal; D, Dye removal; Y, Yes; N, No.

Conclusions sub-section 4. A total of 19 enzymes, out of 46 in the pipeline, have been produced at scales from 1 to 176 grams for validation trials. The partners have already also conducted validation trials carried out with model substrates, model conditions and small scales (from micro- to milliliters). While new enzymes are being produced, and further validated, this report provides the validations performed by Industrial partners, together with additional tests by academic partners.

5. Validations for Wash Performance by Industry Partners: Detergents

A total of 19 enzymes have been produced at scales from 1 to 176 grams for validation trials. Industry partner, Henkel, already completed the analysis of 6 enzymes, namely, EstLip-Dim#008, FE-Polur1, EstLip-PtEst1, Lip9, EstLip-Paes-TB035 and PHE-Paes-PE-H-Y250S. Small-scale enzyme assays were first conducted on those

candidates, with emphasis on *p*-nitrophenyl palmitate (pNPP) activity tests and stability assessments, compared to a benchmark lipase. Table 2 summarizes the specific activity (units/milliliter) of the working enzyme solutions, which were much below those of the benchmark lipase. Table 3 summarizes the residual activity in washing liquor at @40°C +/- protease. As shown, EstLip-Dim-#008, EstLip-PtEst1 and EstLip-Paes-TB035 were the most stable enzymes, retaining from 41 to 114% activity even in the presence of proteases.

Table 2. Specific lipase activity (units/milliliter) of produced enzymes, compared to the benchmark lipase.

Lipase sample	Lipase activity (U/mL)[1]
Benchmark Lipase (IP3461)	71500
EstLip-Dim#008	30
FE-Polur1	90
EstLip-PtEst1	0.2
Lip9 (extract, estimated AEP 30%)	6400
EstLip-Paes-TB035	9
PHE-Paes-PE-H-Y250S	1700

[1]The lyophilised samples were dissolved in 20% PG in the same protein-concentration as a technical benchmark (IP3461), and checked for activity using pNPP

Table 3. Stability in detergent liquor.

Lipase sample	Residual activity[1] at time, t [min]			
	0	20	40	60
Benchmark-Lipase (15µl/L)	100%	101%	107%	109%
Benchmark-Lipase with protease	100%	100%	106%	107%
EstLip-Dim-#008 (0.1ml/5mL)	100%	88%	63%	56%
EstLip-Dim-#008 with protease	100%	76%	48%	41%
FE-Polur1 (0.1ml/5mL)	100%	41%	22%	12%
FE-Polur1 (0.1ml/5mL) with protease	100%	33%	13%	7%
EstLip-PtEst1 (0.2ml/0.4mL)	100%	80%	69%	74%
EstLip-PtEst1 (0.2ml/0.4mL) with protease	100%	80%	69%	74%
Lip9 (CSIC) (0.1ml/5mL)	100%	14%	4%	1%
Lip9 (CSIC) (0.1ml/5mL) with protease	100%	6%	0%	0%
EstLip-Paes-TB035 (200µl/10mL)	100%	98%	106%	111%
EstLip-Paes-TB035 with protease	100%	100%	107%	114%
PHE-Paes-PE-H-Y250S (100µl/50mL)	100%	56%	21%	8%
PHE-Paes-PE-H-Y250S with protease	100%	43%	16%	6%

[1]The lyophilised samples were dissolved in 20% PG in the same protein-concentration as a technical benchmark (IP3461), and checked for activity using pNPP.

Based on the above, washing performance in MWT ("Mini Wash Trials") were set up. Conditions: European detergent w/o enzymes, 6-fold stain (CS46B), 40°C, 1h, 600 rpm. As shown in Table 4, PHE-Paes-PE-H-Y250S and EstLip-PtEst1 did show high washing performance, comparable with that using benchmark BM-lipase, and full-scale wash with PHE-Paes-PE-H-Y250S and EstLip-PtEst1 will be next evaluated.

Table 4. Washing performance in MWT ("Mini Wash Trials").

Lipase sample	Wash performance
BM-Lipase (benchmark)	Wash performance: Yes
EstLip-Dim#008	Poor wash performance
FE-Polur1	Poor wash performance
EstLip-PtEst1	Wash performance: Yes
Lip9	Poor wash performance
EstLip-Paes-TB035	No wash performance, high deviations
PHE-Paes-PE-H-Y250S	Wash performance: Yes

Conclusions sub-section 5. While the validation for all other candidates in Table 1 is ongoing, the results obtained so far indicate that PHE-Paes-PE-H-Y250S and EstLip-PtEst1 lipases are the most promising candidates for full-scale wash, with wash performance comparable with that of benchmark BM-lipase. Optimization loops are proposed for engineered candidates, with potential feedback to engineers. A specific timeframe for full-scale wash trials will be communicated, with initial trials scheduled after the general meeting in May.

6. Validations for Spinning Oils Removal from the Raw Fabrics by Industry Partners: Textiles

A total of 15 enzymes have been produced at scales from 1 to 176 grams for validation trials. Industry partner, Schoeller, already completed the analysis of 4 enzymes, namely, EstLip-Paes-TB035, PHE-Paes-PE-H-Y250S, Lip9 and Polur. Oil removal conditions in Table 5. They were tested for oil removal in 4 materials: 81488 PA/EL 92/8% 180 gr/m^2, 2X34G PES 100% 100 gr/m^2, 5237/00 CO/EL 92/8% 240 gr/m^2, and E03130 PA/EL 80/20% (Table 5). The amount of dye, size, or other fluid by percent weight picked up by the fabric ranged from 47 to 75 depending on the fabrics (Table 6).

Table 5. Spinning Oils Removal in Lab Foulard Machine.

Product	g/L	
Hard water (30ºC)[1]	975	975
Cooking salt	10	10
EstLip-Paes-TB035	15	-
PHE-Paes-PE-H-Y250S	-	15
Total volume (mL)	1000	1000
Foulard	Ja	Ja

Table 6. Materials and Amount of Enzymes Used for Spinning Oils Removal in Lab Foulard Machine.

Material	Pick up (%)	gr Enzyme / Kg Fabrics
81488 PA/EL 92/8% 180 gr/m^2	75	6.4
2X34G PES 100% 100 gr/m^2	60	5.0
5237/00 CO/EL 92/8% 240 gr/m^2	47	5.0
E03130 PA/EL 80/20%	55.8	5.1

To determine the effectiveness of the enzyme treatment to the material, a defined amount of material was extracted in boiling petroleum ether (Table 7) or hot water (Table 8) and the amount in% was determined in relation to the weight of the material. In order to determine the qualitative composition of the application, the extract obtained was measured in the IR (infrared) spectrometer (Table 7). Initial tests were performed using EstLip-Paes-TB035, PHE-Paes-PE-H-Y250S.

Table 7. Quantitative (A.) after extraction with boiling petroleum ether, and Qualitative (B.) percentage of Spinning Oils Removal before and after enzyme treatment in the Lab Foulard Machine.

			Quantitative [%]		B. Qualitative [IR-Spectrum]	
Nr. Article	**Condition**	**Enzyme**	**A. Before**	**A. After**	**B. Before**	**B. After**
3X58 100% PES	Raw	Est.Lip Paes TB 035	1.6	1.5	Fatty acid ester, mineral oil, paraffin	Paraffin, fatty acid ester, amidelittle silicone
	Pretreated	PEH-Paes PEH Y250S	0.0	1.4	Quantity too small for analysis	Paraffin, fatty acid ester, amidelittle silicone
E03130 80%PA6, 20%EL	Raw	Est.Lip Paes TB 035	1.9	1.3	Fatty acid ethoxylate, amide, silicone	fatty acid esters, silicone
	Pretreated	PEH-Paes PEH Y250S	0.1	1.4	Quantity too small for analysis	Fatty acid esters (ethoxylate), little silicone
5237-00 92% CO, 8% EL	Raw	Est.Lip Paes TB 035	0.7	0.8	Fatty acid esters, silicone	Paraffin, silicone, little fatty acid ester, amide
	Pretreated	PEH-Paes PEH Y250S	0.5	0.7	Fatty acid esters, silicone	Paraffin, silicone, little fatty acid ester, amide
61488F1 92% PA, 8% EL	Raw	Est.Lip Paes TB 035	2.6	1.8	Fatty acid ethoxylate, amide (low amount)	Mineral oil, silicone, little fatty acid ester
	Pretreated	PEH-Paes PEH Y250S	0.2	1.8	Fatty acid ester, polyamide, silicone	Mineral oil, fatty acid esters, silicone

Table 8. Quantitative (A.) after extraction with hot water, and Qualitative (B.) percentage of Spinning Oils Removal before and after enzyme treatment in the Lab Foulard Machine.

			Quantitative [%]		B. Qualitative [IR-Spectrum]	
Nr.Article	**Condition**	**Enzyme**	**C. Before**	**C. After**	**B. Before**	**B. After**
3X58 100% PES	Raw	Est.Lip Paes TB 035	0.2	1.5	Unknown substance, ester, amide and carboxylate (e.g. CMC, alginate, etc.)	carboxylic acid salt, carbonate, amide, unknown
	Pretreated	PEH-Paes PEH Y250S	0.2	0.5	Polycarboxylate (e.g. CMC)	PES, carbonate, carboxylic acid salt
E03130 80%PA6, 20%EL	raw	Est.Lip Paes TB 035	0.3	0.5	Polyamide	Polyamide, silicone
	Pretreated	PEH-Paes PEH Y250S	0.4	0.6	Polyamide	Polyamide, silicone
5237-00 92% CO, 8% EL	Raw	Est.Lip Paes TB 035	8.8	0.7	Polycarboxylate (e.g. CMC, alginate or similar)	Carboxylic acid salt[1]
	Pretreated	PEH-Paes PEH Y250S	0.2	0.8	Polycarboxylate (e.g. CMC, alginate or similar)	Carboxylic acid salt[1]
61488F1 92% PA, 8% EL	raw	Est.Lip Paes TB 035	0.5	0.7	Polyamide	Polyamide
	Pretreated	PEH-Paes PEH Y250S	0.2	0.8	Polyamide	Polyamide

[1]Possibly carboxymethyl tamarind

Other set of experiments were performed with Lip9, Polur1 and PEH-Paes PEH Y250S. Although PEH-Paes PEH Y250S had already been used in previous trials (see Table 7, Table 8), a new trial was conducted to evaluate the possible effect of pH control or lack thereof during the washing process. Control tests without enzymes were also performed. Experimental set up and materials and enzyme amount used are summarized in Table 9 and Table 10. Each sample run through the lab Foulard twice, and before and after the treatment, the pH was measured (Table 11), to evaluate whether any effect of the removal of spinning oils is detected. The results of these experiments are shown in Table 12 and Table 13, and were compared to a control experiment with no enzyme used (Table 14) and compared to the Original Schoeller treatment (extensive washing with hot water) (Table 15).

Table 9. Spinning Oils Removal in Lab Foulard Machine.

Hard water[1]	989.75	986.00	970.00	990.00	pH-enzyme solution
Cooking salt	0.00	0.00	0.00	0.00	-
PHE-Paes-PE-H-Y250S	0.25	-	-	-	7.5
Lip9	-	4.00	-	-	7.5
Polur1	-	-	20.00	-	7.0
HEPES buffer	10.00	10.00	10.00	10.00	7.5
Ammonia 24%					-
Total volume (mL)	1000	1000	1000	1000	-
Foulard	Ja	Ja	Ja	Ja	-

[1]30ºC

Table 10. Materials and Amount of Enzymes Used for Spinning Oils Removal in Lab Foulard Machine.

Material	Pick up (%)	gr Enzyme / Kg Fabrics
61488Z PA/EL 92/8% 180 gr/m^2	75	6.4
2X34G PES 100% 100 gr/m^2	60	5.0
E03130 PA/EL 80/20%	55.8	5.1

Table 11. pH-measurements.

Medium	**Liquid**	**On sample surface after treatment**	**On sample surface after 24 hours**
Water	7.2	-	-
Water+HEPES	6.5	-	-
Water+HEPES+40 drops of ammonia	7.5	-	-
PEH-Paes PEH Y250S cocktail	7.5	7.5	7.5
Lip9 cocktail	7.5	7.5	7.5
Polur 1 cocktail	7	7	7.0

As shown in Table 15 the Original Schoeller treatment (extensive washing with hot water) extensively eliminated all spinning oils, reducing their content from 1.6-2.6% in the raw material to 0-0.2%. The elimination levels targeted were not attained by any of the enzymes, and in some cases, there was no removal of spinning oils present in the raw fabric. For instance, in the case of Lip9, small reductions were detected in 3X58 100% PES (polyester), ranging from 0.2% to no residue. However, further testing is needed to confirm these reductions. Although, we had difficulties to stabilize the pH above 7 in initial tests, which was recommended. This is why we added some ammonium to try to control pH after each step. Then, given that no variations in the pH of the solution were observed during the treatment, we consider that the enzymes have not delivered any desired major effect not due to enzyme instability caused by process performance (pH). Instead, it is possible that the time frame in the Foulard machine may be too short for the enzymes to take effect. Since the procedure applied is the most realistic one from an industrial point of view, trials will continue with other enzymes that have already been supplied.

Table 12. Quantitative (A.) after extraction with boiling petroleum ether, and Qualitative (B.) percentage of Spinning Oils Removal before and after enzyme treatment, without buffer, in the Lab Foulard Machine.

Nr. Article	1-Est.Lip Paes TB 035		2-PEH-Paes PEH Y250S	
	A.[%][1]	B.[1]	A.[%][1]	B.[1]
3X58 100% PES	1.5	Paraffin, fatty acid ester, amidelittle silicone	1.4	Paraffin, fatty acid ester, fatty acid amide, little silicone
E03130 0%PA6, 20%EL	1.3	Fatty acid esters, silicone	1.4	fatty acid esters (ethoxylate), little silicone
61488F1 92% PA, 8% EL	1.8	Mineral oil, silicone, little fatty acid ester	1.8	mineral oil, fatty acid esters, silicone

[1]A. Quantitative; B. Qualitative [IR-Spectrum].

Table 13. Quantitative (A.) after extraction with boiling petroleum ether, and Qualitative (B.) percentage of Spinning Oils Removal before and after enzyme treatment with HEPES buffer, in the Lab Foulard Machine.

Nr. Article	3-PEH-Paes PEH Y250S		4-Lip9		5-Polur 1	
	A.[%][1]	B.[1]	A.[%][1]	B.[1]	A.[%][1]	B.[1]
3X58 100% PES	0.2	Fatty acid esters, possibly paraffin, silicone	0	No residue	0.2	Paraffin, fatty acid ester/amide, PES, silicone
E03130 0%PA6, 20%EL	1.4	Possibly polyurethane	1.2	Possibly polyurethane	0.9	Fatty acid (ethoxylate), silicone, possibly a little PES
61488F1 92% PA, 8% EL	1.2	Fatty acid ethoxylate, little silicone	1.2	Fatty acid esters, silicone	0.9	Mineral oil, silicone, fatty acid ester/amide, low PES

[1]A. Quantitative; B. Qualitative [IR-Spectrum].

Table 14. Quantitative (A.) after extraction with boiling petroleum ether, and Qualitative (B.) percentage of Spinning Oils Removal before and after enzyme treatment with HEPES buffer, in the Lab Foulard Machine.

Nr. Article	6-Control-Cocktail without Enzyme	
	A.[%][1]	B.[1]
3X58 100% PES	0.3	Paraffin, PES, fatty acid amide, silicone
E03130 0%PA6, 20%EL	1.4	Possibly polyurethane
61488F1 92% PA, 8% EL	1.1	Fatty acid esters, silicone

[1]A. Quantitative; B. Qualitative [IR-Spectrum].

Table 15. Quantitative (A.) after extraction with boiling petroleum ether, and Qualitative (B.) percentage of Spinning Oils Removal before and after Original Schoeller treatment in the Lab Foulard Machine.

Nr. Article	**Raw**		**Original Schoeller Treatment**	
	A.[%][1]	B.[1]	A.[%][1]	B.[1]
3X58 100% PES	1.6	Fatty acid ester or emulsified mineral oil or paraffin	0	Quantity too small for analysis
E03130 0%PA6, 20%EL	1.9	Fatty acid ethoxylate, amide, silicone	0.1	Quantity too small for analysis
61488F1 92% PA, 8% EL	2.6	Fatty acid ethoxylate, amide (little amount)	0.2	Fatty acid ester, polyamide, silicone

[1]A. Quantitative; B. Qualitative [IR-Spectrum].

Conclusions sub-section 6. While the original Schoeller treatment effectively removes spinning oils from raw fabric, the enzymes tested in this study did not achieve similar levels of elimination. That said, additional candidates in Table 1 are being tested. Despite the lack of significant results, it's suggested that the short time frame in the Foulard machine might have limited the effectiveness of the enzymes rather than enzyme instability due to pH variations. The decision to continue trials with other supplied enzymes acknowledges the need for further exploration to find an enzyme or combination of enzymes that can effectively address the issue of spinning oil removal in a realistic industrial setting. A specific timeframe for full-scale trials will be communicated, with initial trials scheduled after the general meeting in May. In addition to that Optimization loops are proposed for engineered candidates, with potential feedback to engineers. A specific timeframe for full-scale wash trials will be communicated, with initial trials scheduled after the general meeting in May. As example, engineering of enzymes for textiles application were undertaken by partner INOFEA with the main objective of producing improved and stable enzymes by applying a supramolecular engineering approach, by applying the technology of enzyme immobilization and shielding.

7. Validations for Hydrolysis of Hyaluronic Acid by Industry Partners: Cosmetics

Hyaluronic acid substrate A) HA raw 600-900kDa, and B) HA50 20-30kDa, supplied by the industrial partner Evonik, were used (Fig. 8).

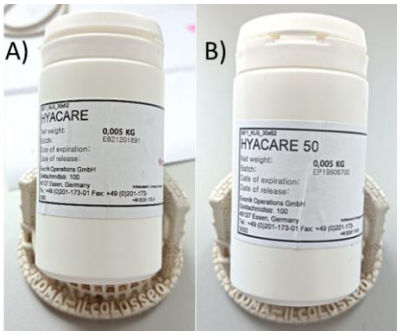

Fig. 8. Hyaluronic acid as substrate A) HAraw 600-900kDa, B) HA50 20-30kDa.

Small-scale reaction conditions for Hyalo-oligos production were initially set up. For that, the enzymatic reaction mixture consisted of 100 µL of the hyaluronidase (HAase) supplied by BioSynth (Hyal_HRDSV_2334) and a 2 mg/mL HA (Fig.1) solution in H_2O. After incubation at 35 °C for an appropriate time, the reaction mixture was inactivated by incubating the samples in a Thermomixer (Eppendorf, Hamburg, Germany) for 5 min at 95 °C. Time course samples were analyzed by the Dinitro Salicilic Acid (DNS) colorimetric assay and in order to see in which time we had to stop the reaction to obtain the desired molecular weight range Hyalo oligos (≤ 5kDa), samples were diluted with water (1:10) and then analyzed using high performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) on an ICS3000 Dionex system consisting of a SP gradient pump, an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode, an auto-sampler (model AS-HV). All eluents were degassed by flushing with helium. An anion-exchange 4 x 250 mm Carbo-Pack PA-100 column (Dionex) connected to a 4 x 50 CarboPac PA-100 guard column were used at 30 °C. Eluent preparation was performed with Milli-Q water, 50% (w/v) NaOH and sodium acetate trihydrate. The compounds were eluted by a gradient method in which the initial mobile phase was 30 mM NaOH and 270mM of sodium acetate trihydrate for 45 min, then from minute 45 to minute 70, the mobile phase was increased gradually until it reached a concentration of 100 mM NaOH and 900 mM of sodium acetate, which was further maintained during 5 min. Finally, initial conditions were reset and maintained for 10 min to equilibrate the column, using a flow rate of 0.5 mL/min for. The peaks were analyzed using Chromeleon software (Dionex, Thermo Fischer Scientific Inc., Waltham, MA, USA). Fig. 9 illustrates the degradation product profile obtained after hydrolyzing HA raw 600-900kDa and HA50 20-30kDa at a concentration of 2 mg/mL (or 0.2% w/v) using Hyal_HRDSV_2334, following a 4-hour incubation at 35 °C. The percentage of degradation was nearly 100%, as no high molecular weight HA was detected under our analysis conditions.

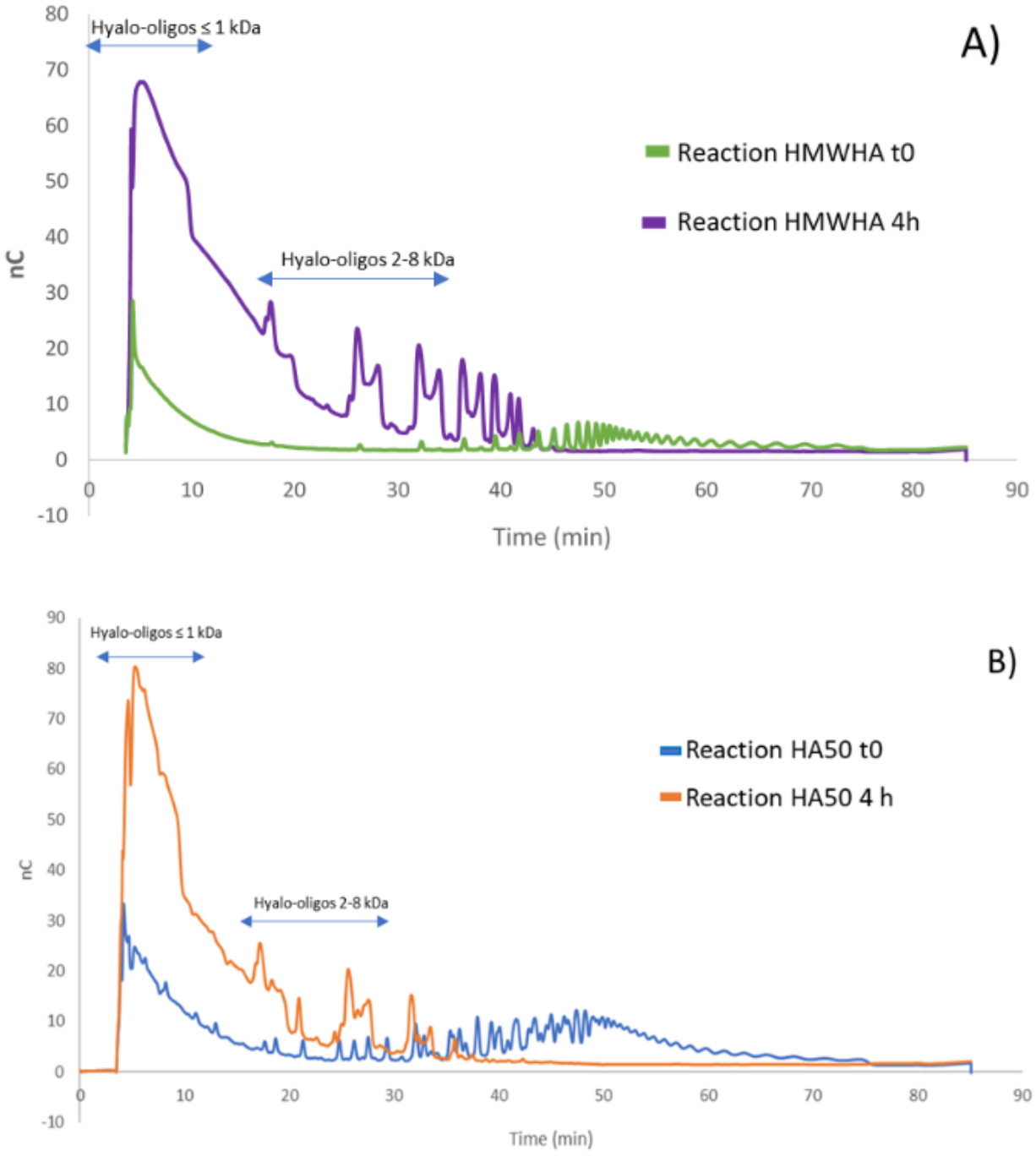

Fig. 9. HPAEC-PAD chromatograms showing the breakdown of A) high-molecular-weight hyaluronic acid (HMWHA) and B) HA 50, reaction mixture consisted of 100 µL of the HAase and 2 mg/mL HA solution in H_2O reaction final volume 1 mL; the incubation was at 35 °C for 4 h.

Industrial partner Evonik confirmed that the molecular weight (MW) of the final hydrolysis products should be below 5 kDa, preferably 1-2 kDa. A detailed inspection of the hydrolysis products shown in Fig. 8 demonstrates that the hyaluronidase cleaves both HA raw materials into hydrolytic products of the desired size. Based on peak areas, it can be inferred that the 1-2 kDa products represent approximately 90% of the total degradation products when employing HA raw 600-900 kDa or HA50 20-30 kDa, and amount that increase to about 95% when considering products below 5 kDa. We are currently performing optimization of some reaction parameters to produce a reaction product with desired size. The reaction scaling, once optimized, will be carried out preserving the proportions and conditions previously stipulated. After

incubation and inactivation, the products will be analyzed by HPAECPAD to ensure that we have the desired range of hyalo-oligos. Subsequently, a semi-purification of compounds will be carried out using membranes of different pore sizes and finally the sample will be lyophilized. Liophilization will also be performed after reaction without any semi-purification steps. Industrial partner Evonik is interested in using HA raw 600-900 kDa as starting material rather than HA50 20-30 kDa because production costs, and this is why optimization of reaction conditions for producing the desired hydrolytic products will be performed with this material. In total we need at least 10- 25 g of such hydrolysis products. For Gel Permeation Chromatography (GPC) and for the first Bioactivity screening a total of 1-2 g of such lyophilized hydrolysis products or dried powder (> 90% active substance) are going to be produced. Once produced, to evaluate the hydrolyzed HA, the following activities and tests have been planned, at Evonik.

1) Quality of the product: the quality of the product will be investigated by measuring the MW and polydispersity, pH, salts of Hyaluronate, glucuronic Acid and protein.
2) Cosmetic formulation: The product will be incorporated into different cosmetic formulations and the compatibility and the stability in the formulation systems will be tested.
3) Bioactivity and efficacy: The efficacy of the hydrolyzed HA will be evaluated by skin penetration study and by SimDerma® screening platform, which is an in vitro multiparametric platform that includes multiple experiments related to 17 key cosmetic targets and clustered in 7 skin claims. In addition, depending on the bioactivity and availability of the skin Panel, the formulations will be tested on skin after different application times, by performing trans epidermal water loss (TEWL) and skin hydration (Corneometer) tests.
4) TOX Evaluation: Skin sensitization evaluation will be performed by read across of Evonik HA products.

Conclusions sub-section 7. While other hyaluronidases are being tested, we found one named Hyal_HRDSV_2334, which seems to be a priority target for producing hyaluronic products for their integration into Evonik cosmetics. Based on the results obtained, it's evident that the hyaluronidase effectively produces hydrolytic products within the desired size range, meeting Evonik's specifications, at low temperatures (35ºC). Approximately 90-95% of the degradation products fall within the 1-2 kDa range, demonstrating the enzyme's efficacy. Ongoing optimization of reaction parameters aims to ensure consistent product quality, with a focus on using HA raw 600-900kDa due to its cost advantage according to Evonik's preferences. Production targets are set at 10-25g of hydrolysis products, while testing will encompass quality assessment, formulation compatibility, bioactivity, efficacy, and TOX evaluation. These findings inform our next steps toward delivering hydrolyzed HA products tailored to Evonik's requirements.

8. Validations by Academic Partners

The trials conducted by the industrial partners summarized in Sections 5-7 were preceded by a prior confirmation, carried out by the academic partners, of the activities of each enzyme against substrates, model conditions and small scales (from micro- to milliliters). The results of these confirmations are documented in Deliverables D6.1 (Best 18 Pre-Lead Enzyme Materials Obtained at Gram Scale for Real-Life Tests) and D6.2 (Report on Fermentation, DSP and Activity Verification for 18 Prelead Enzymes), submitted in January 2024. Within the framework of Deliverable D7.1, the academic partners have also conducted validation trials aimed at identifying suitable reaction conditions for the industrial partners. Below are the most pertinent results, utilizing enzymatic materials supplied by BioSynth as detailed in Table 1.

- Detergent applications

IST-ID partner contributed by validating the activity and performance of the following enzyme materials, for detergent applications: PEH_Pbau_PE-H, PEH_PForm_PE-H, PEH_Poco_PE-H, PstLip_TBEc304, FE_EH37 and GEN0105. Initial assays to compare enzyme performance were conducted in 96-well plates using tributyrin and trioctanoin as substrates in the absence and presence of wash liquour (Fig. 10). Under the conditions tested, PEH_Poce_PE-H showed the highest activity for both substrates in the presence of wash liquour.

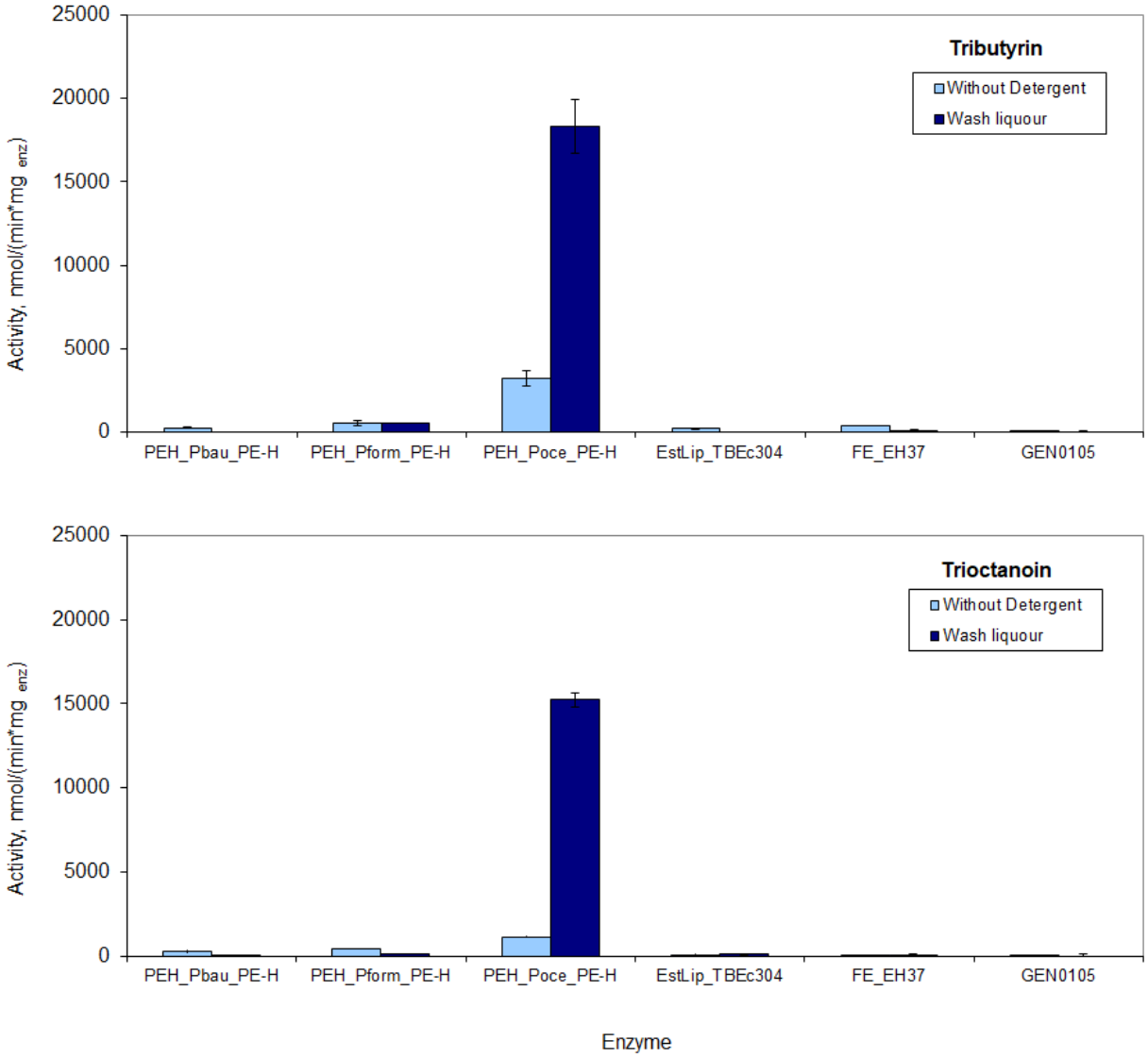

Fig. 10. Enzyme activity using tributyrin (upper panel) and trioctanoin (lower panel) as substrate. Assays conducted in microtitre plates at 30°C and 200 rpm, with 200 µL of reaction medium per well containing the following: 90.9 µL EPPS 2.5 mM pH 8 buffer; 9.09 µL enzyme solution at 0.5-5 mg/mL; substrate 100 mM in DMSO; 90.9 µL phenol red 0.304 mM in EPPS buffer; with/without 3.1 g/L detergent (Henkel).

To assess the efficiency of the selected enzymes, assays in 10 mL glass reactors were carried out under the following 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, 800 rpm (Fig. 11). The fatty acid composition of the beef fat was analysed before and after enzymatic reaction by gas-chromatography (using flame ionization detection, GC-FID, and mass spectrometry, GC-MS). Additionally, the enzymes were tested in the presence of wash liquour (3.1 g/L detergent in Tris-HCl buffer) to mimic washing conditions (Fig. 12). After 24h of conversion, the enzyme PEH_Poce_PE-H had converted between 18 and 66% of each fatty acid present in the beef fat soiled cloth. In general, saturated fatty acids such as 14:0, 16:0 and 18:0 were converted at lower rate than unsaturated fatty acids such as 16:1n7 and 18:1n9.

Fig. 11. Small scale reactors used for testing enzyme activity with soiled cloths.

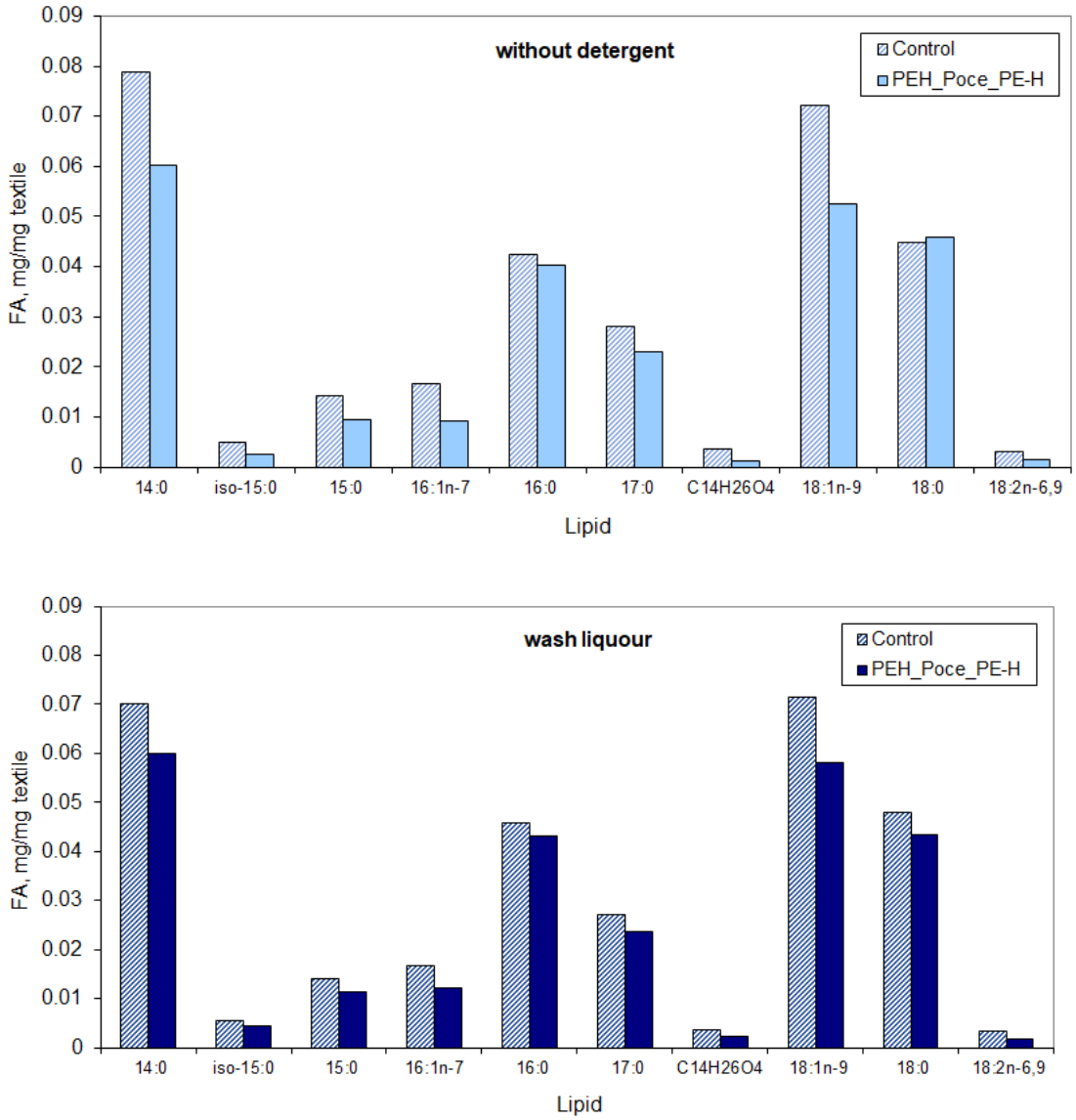

Fig. 12. Fatty acid converted per mg of soiled cloth by PEH_Poce_PE-H in the small scale reactors shown in Fig. 9, in the absence (upper panel) and presence (lower panel) of wash liquour.

When the effect of calcium on enzyme activity was studied, it was found that 50 mM $CaCl_2$ could favour enzyme activity (Fig. 13).

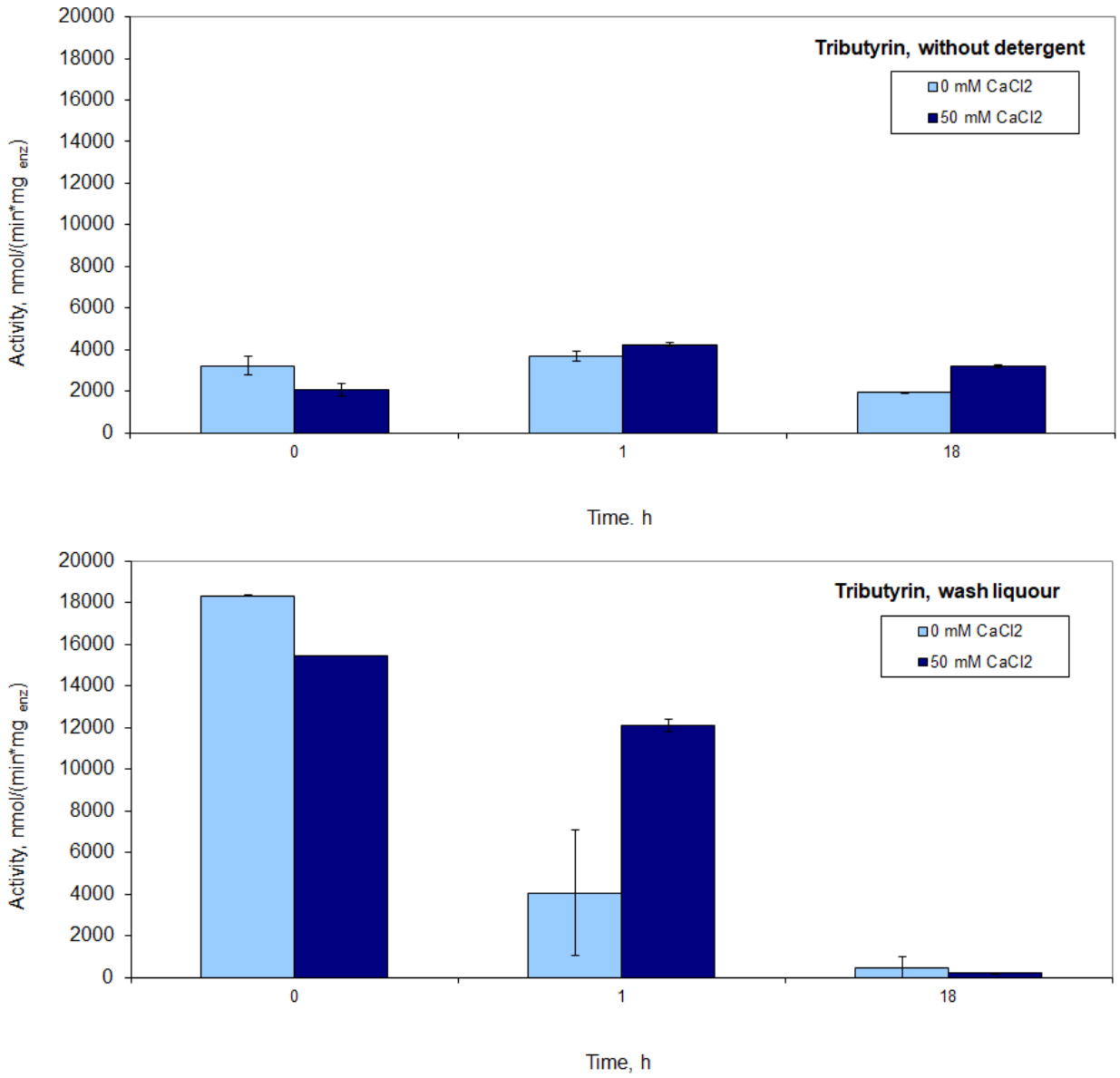

Fig. 13. Effect of calcium chloride on the activity of PEH_Poce_PE-H converting tributyrin (upper panel) and trioctanoin (lower panel).

In fact, most of the tested enzymes presented higher activity when $CaCl_2$ was added to the reaction media, and with EstLip_Paes_TB035, EstLip_Dim_#08, PEH_Pbau_PE-H, FE_Polur1, Lip9 Val161Ser, and PEH_Pform_PE-H, $CaCl_2$ increased significantly enzyme activity in wash liquour (Fig. 14). Curiously, the highest activity in washing liquour was attained for EstLip_TBEc304 converting tributyrin (34611.16 nmol min^{-1} mg_{enzyme}^{-1}) in the absence of calcium, and for PEH_Pbau_PE-H converting trioctanoin (35945.54 nmol min^{-1} mg_{enzyme}^{-1}) in the presence of 50 mM $CaCl_2$.

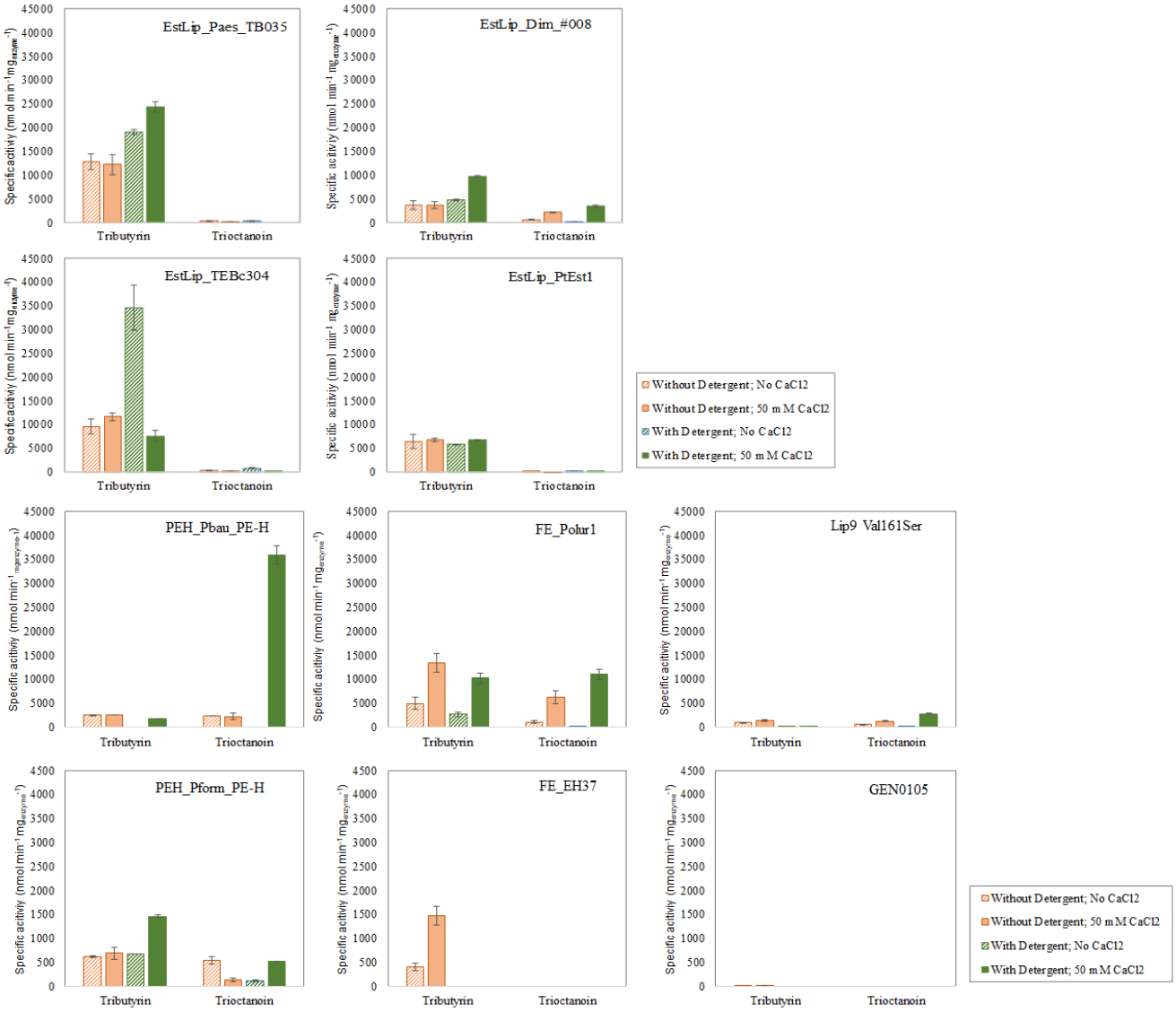

Fig. 14. Effect of 50 mM $CaCl_2$ on enzyme activity in the absence and presence of wash liquour.

CSIC partner contributed validating the activity and performance of the following enzyme materials, for detergent applications: Lip9 Val161Ser mutant and FE_EH_{37}.

Lip9 Val161Ser was computationally designed by partner CSIC to improve thermostability and its activity versus long chain esters. The data shown in Table 16 demonstrated that the mutant was about 65-fold more active than the wildtype enzyme, as well as from 3 to 14-fold more stable at temperatures from 30 to 50ºC. Once confirmed, the washing capacity for stained clothes was further evaluated at 30ºC. As shown in Fig. 15, Lip9 Val161Ser CSIC released more fatty acids from all the stained clothes of HENKEL than Lip9 WT (wild type). After all these experiments we conclude that this mutant might be more appropriate for washing tests by industry partner Henkel compared to the wild type Lip9.

Table 16. Relative activity and thermostability, by meaning of half-life time, of Lip9 Val161Ser, compared to Lip9.

Temperature (°C)	**Specific activity (U/mg)[1]**		**Half-life time, T_{50} (h)**	
			Lip9 WT	Lip9 Val161Ser
30	0.21	1.3	27.22 ± 0.61 h	77.57 ± 0.15 h
40	-	-	1.33 ± 0.01 h	18.18 ± 0.04 h
50	-	-	1.35 ± 0.001 min	17.95 ± 0.02 min

[1]Calculated with Phenol Red assay using tributyrin as substrate at 30°C and pH 8.0.

[2]Calculated as follows: [Enzyme], 0.8 µg mL; [p-nitrophenyl butyrate, p-NPB], 2 mM; reaction volume, 100 µl HEPES, buffer, 40 mM, pH 7,0; T, 30°C, 40°C or 50°C; assay format, 96-well plates (ref. 655101, Greiner Bio-One GmbH, Kremsmünster, Austria); assay wavelength, 348 nm. Datasets were collected with a Synergy HT Multi-Mode Microplate reader (with Gen5 2.00 software Biotek Instruments, Winooski, VT, USA), with values obtained from the best linear fit using Excel 2019. All reactions and analyses were performed in triplicate (n = 3).

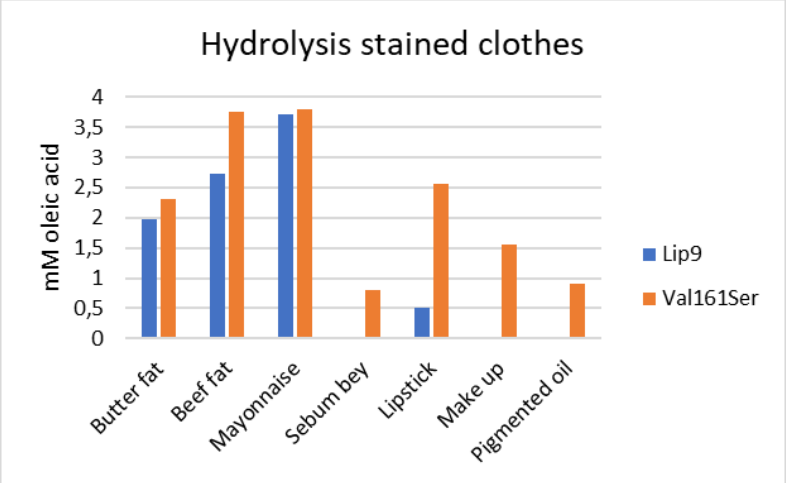

Fig. 15. Concentration (mM) of free fatty acids released from stained clothes. The results show the comparison of fatty acid release between Lip9 and Lip9 Val161Ser from CSIC. Reaction conditions: 4 mg of stained cloth; 0.05 mg of enzyme in 100 µL of Washing liquor buffer (2.5 g/L) at 30°C, pH 8.0, 2 hours.

The EH_{37} enzyme material provided by BioSynth was further tested to validate its hydrolytic activity. As shown in Fig. 16, the enzyme was quite active, but no activity for triglycerides longer than tributyrin was confirmed, limiting its application in detergent applications.

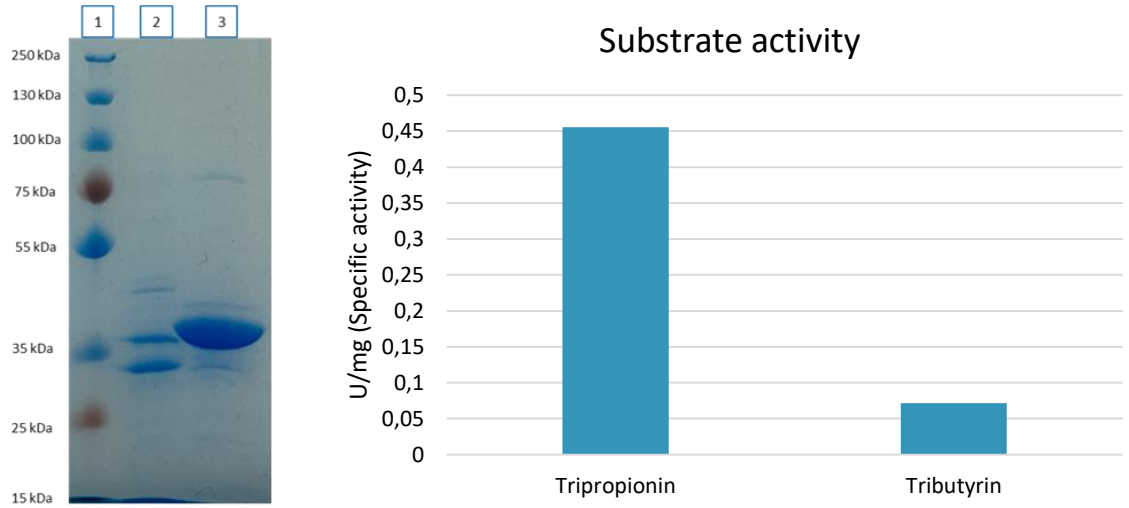

Fig. 16. Characteristics of EH37. A**.** SDS-PAGE analysis. A 12% SDS-polyacrylamide gel is shown. Lane 1. Molecular weight marker. Lane2. EH37 produced by Biosynth in *Pichia* Lane3. EH37 CSIC pure enzyme produced in *E. coli*. **B**. Specific activity (U/mg) of EH37 from Biosynth with tripropionin and tributyrin at pH 7.0 and 30°C.

Conclusions sub-section 8 - detergent. The partners validated enzyme materials for detergent applications, with PEH_Poce_PE-H showing high activity in the presence of wash liquor. Calcium enhanced enzyme activity, particularly for EstLip_Paes_TB035 and PEH_Pbau_PE-H. CSIC validated Lip9 Val161Ser mutant, demonstrating significantly higher activity and stability compared to the wild type enzyme, making it potentially more suitable for washing tests by industry partner Henkel. This should be taken into consideration for applications in industrial-scale detergent formulations and tests.

- Cosmetic applications

The activity of the enzymes Hyal_HRDSV_2334 and *Vibrio* sp. IAMC-CNR#23 were tested in 24 well microtiter plates, at 30°C and 200 rpm, containing the following per well: 900 µL of a 2 g/L solution of Hyacare (HA) or Hyacare 50 (HA50) from Evonik in phosphate buffer pH 6.2; enzyme at a concentration of 1, 20 or 40 g/L. Both enzymes degraded HA50 at a higher rate than HA and increased degradation was observed with increasing enzyme concentration (Fig. 17).

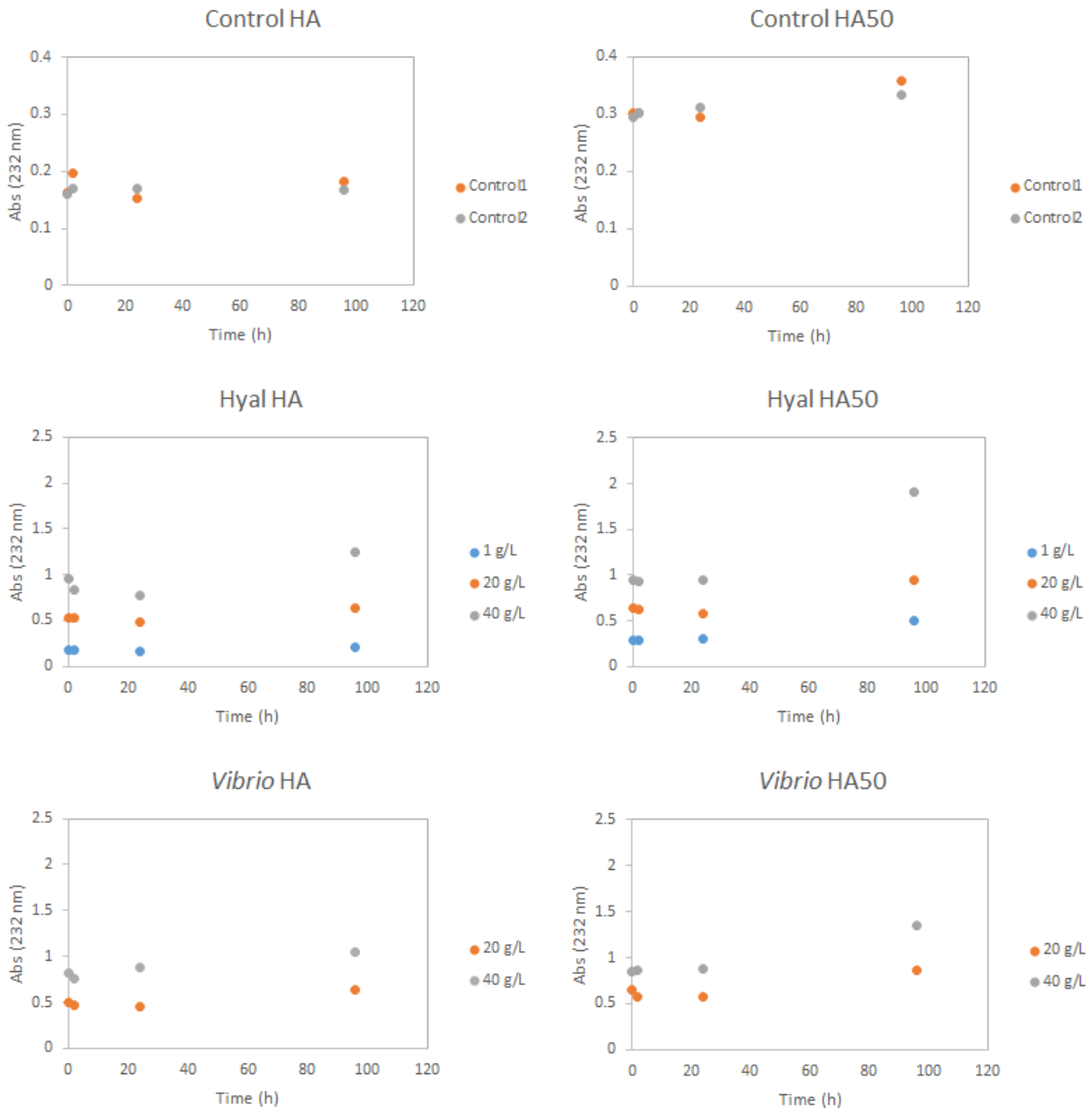

Fig. 17. Activity of the hyaluronidases Hyal_HRDSV_2334 and *Vibrio* sp. IAMC-CNR#23 when using Ha and HA50 as substrates. Control assays contained no enzymes.

When the bioconversion of 2g/L HA50 was carried out in 10 mL glass reactors at 30 and 37°C (Fig. 18), *Vibrio* sp. IAMC-CNR#23 presented higher activity at 30°C than at 37°C (Fig. 19).

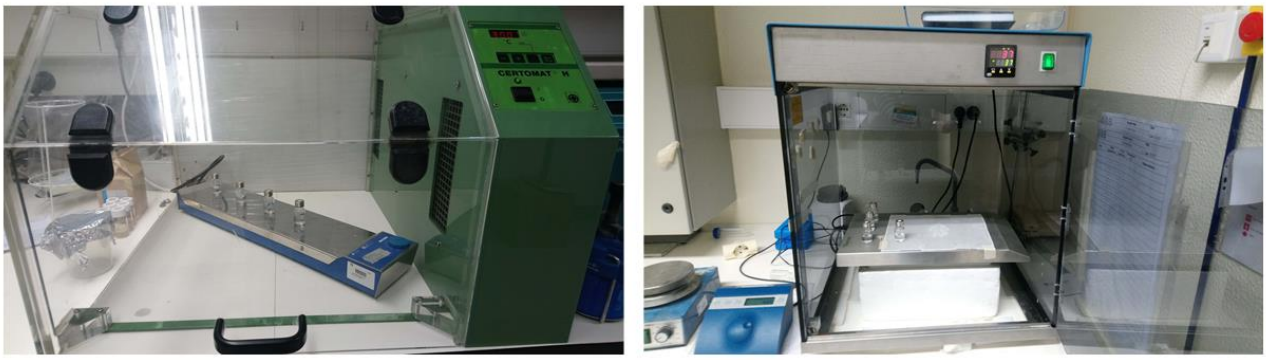

Fig. 18. Bioconversion of HA50 in small scale reactors at 30°C (left) and 37°C (right).

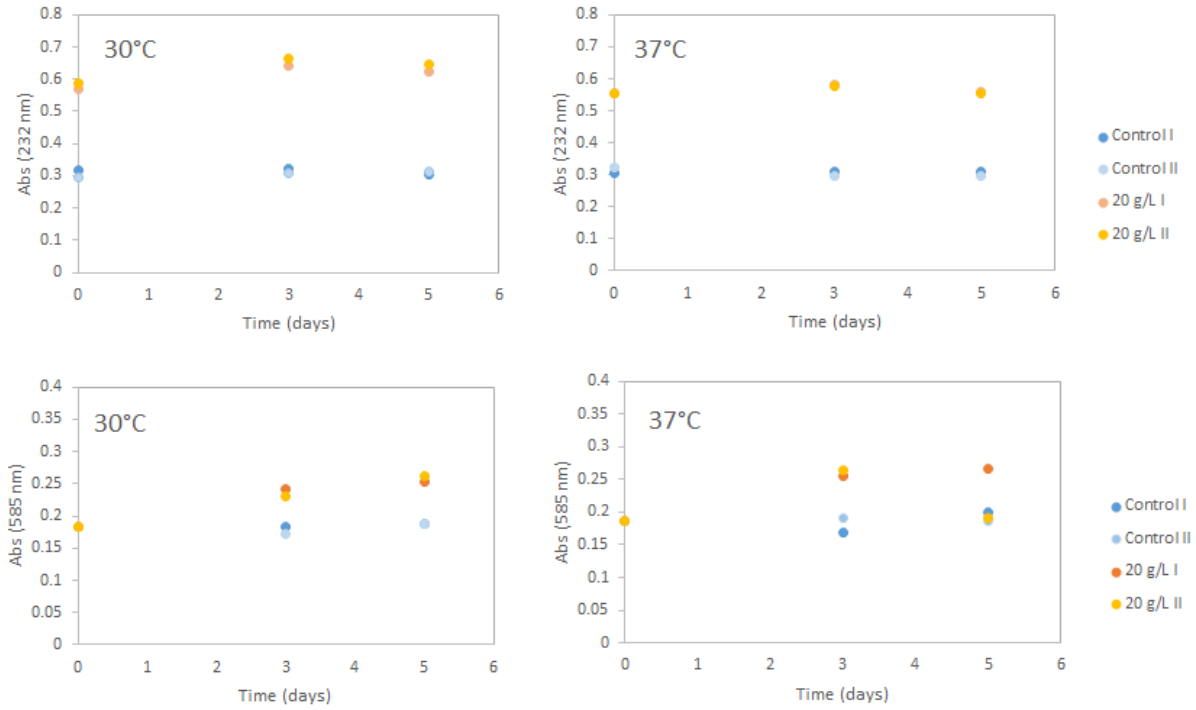

Fig. 19. Hyaluronidase activity of *Vibrio* sp. IAMC-CNR#23 using 2 g/L HA50 at 30 and 37°C. The enzyme concentration was 20 g/L. Control assays contained no enzyme.

Once more, when $CaCl_2$ was added to the reaction medium, enzyme activity was significantly improved (Fig. 20). Simultaneous addition of BSA and $CaCl_2$ further increased enzyme activity.

Conclusions sub-section 8 - cosmetics. The activity of enzymes Hyal_HRDSV_2334 and *Vibrio* sp. IAMC-CNR#23 was assessed in 24 well microtiter plates, with both showing higher degradation rates of HA50 compared to HA, and degradation increased with enzyme concentration. Additionally, in 10 mL glass reactors, *Vibrio* sp. IAMC-CNR#23 exhibited higher activity at 30°C compared to 37°C and in the presence of calcium. These findings suggest temperature dependence in enzyme activity, with implications for bioconversion processes involving HA and HA50 substrates. The results, at small scales, with Hyal_HRDSV_2334 confirmed the results detailed in Section 6, and the potential application of this hyaluronidase for cosmetic applications. In addition, partner Chem_Sol might consider degradation tests of hyaluronidase Vibrio sp. IAMC-CNR#23 at 30ºC.

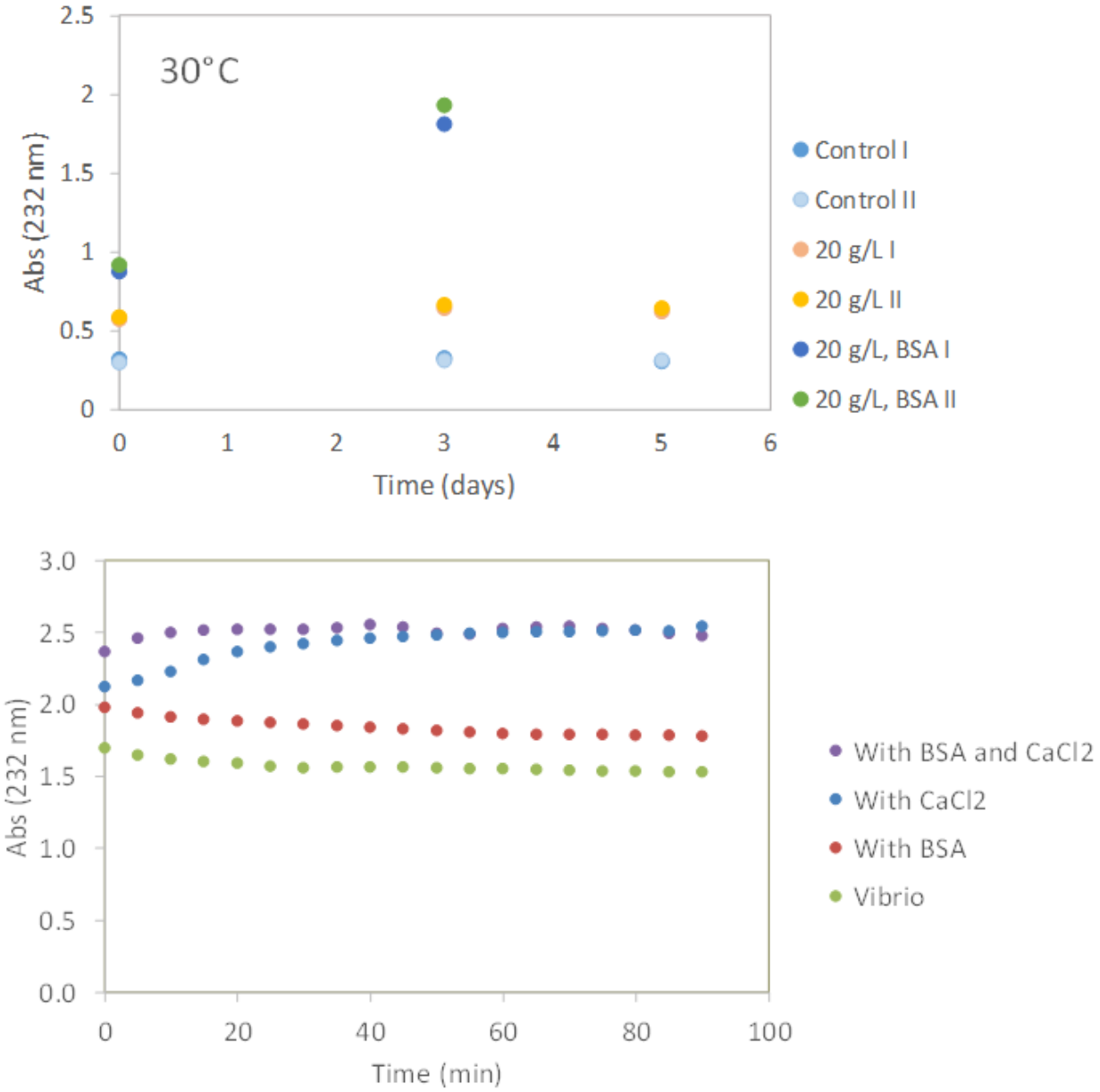

Fig. 20. Effect of BSA and calcium on hyaluronidase activity of *Vibrio* sp. IAMC-CNR#23 using 2 g/L HA50 at 30°C. To prevent precipitation, in the lower panel, the reaction was carried out using MOPS as buffer.

- Textiles application

The spinning oils of the fabrics provided by Schoeller were removed using all available enzymes, under the conditions suggested to mimic the industrial treatment: 200% of the weight of fabric was added as solution of enzyme at 12.5 g/L in TRIS-HCl 100 mM pH 8. The biodegradation was carried out at 30ºC for 24h and at the end the fabrics were washed once with 1 g/L genapol X-100 and once with Milli-Q water.

Several enzymes were effective in removing some compounds present in the fabrics 61488, 3X58, and 5237 (Fig. 21). Depending on the type of fabric, they contained fatty acids, alkanes, phenols and compounds that could not be identified by GC-MS. The enzymes tested were more effective in degrading the fatty acids (Fig. 22). Nevertheless, the level of the chemically treated fabrics was never attained under the conditions tested.

Fig. 21. Composition of the spinning oils in the fabrics 61488 (top), 3X58 (middle), 5237 (bottom) without treatment, after chemical treatment (at Schoeller), and after enzymatic treatment. The area of the peaks was corrected using an internal standard and normalized using the weight of fabric used.

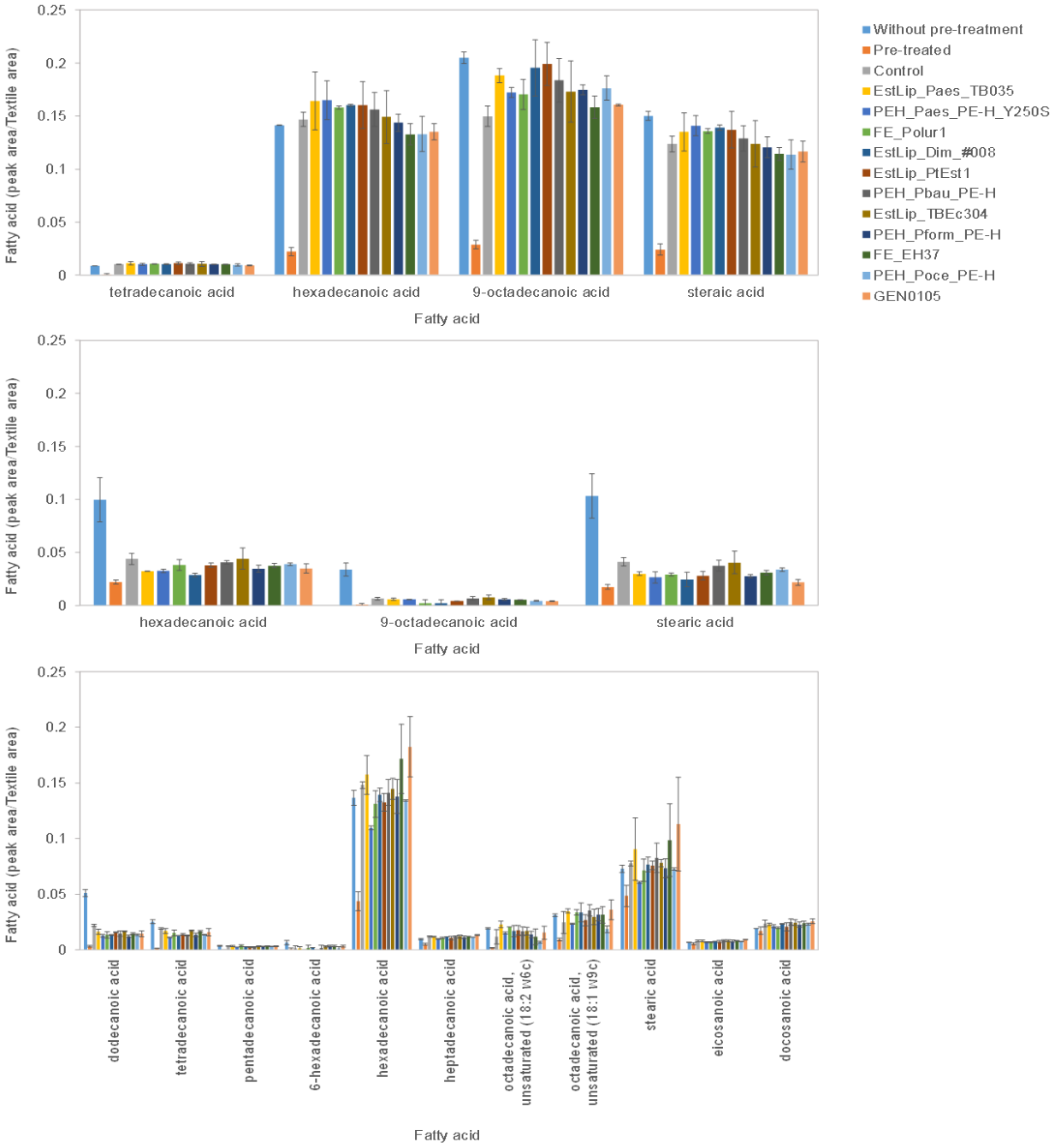

Fig. 22. Composition in fatty acids of the spinning oils in the fabrics 61488 (top), 3X58 (middle), 5237 (bottom) without treatment, after chemical treatment (at Schoeller), and after enzymatic treatment. The area of the peaks was corrected using an internal standard and normalized using the weight of fabric used.

The enzyme Lip9 Val161Ser and the bacterial strains *Psychrobacter celer*, *Pseudomonas protegens,* and *Serratia quinivorans* were also tested under the same conditions to remove the spinning oils from the same fabrics. Once more, good results could be observed with some compounds, and in particular fatty acids, but the level of removal was below that attained with the current industrial treatment (Fig. 23).

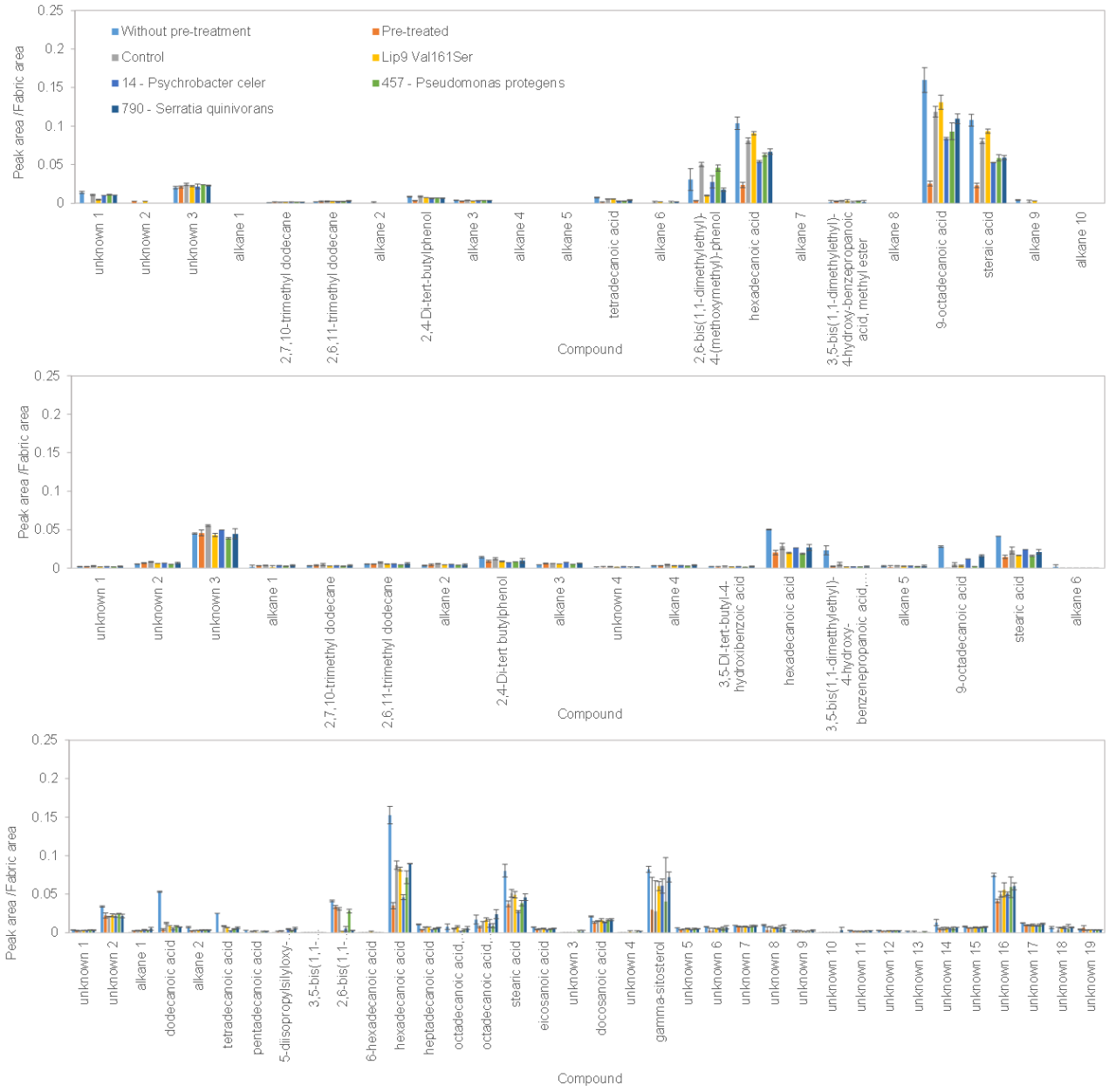

Fig. 23. Composition of the spinning oils in the fabrics 61488 (top), 3X58 (middle), 5237 (bottom) without treatment, after chemical treatment (at Schoeller), and after enzymatic treatment. The area of the peaks was corrected using an internal standard and normalized using the weight of fabric used.

The dyeing liquid after the dyeing process carried out at Schoeller, contains several compounds that confer a grey to black colour to the liquid. The ability of the enzyme Pvec11 to clean the dyeing liquid was assessed in microtitre plates and small-scale reactors (Fig. 24). The microtitre plate allowed the monitoring of the enzymatic reaction in real time for 1h by a UV-VIS spectrophotometer. The initial and end point (1h) of the reaction for 5, 10 and 20% of the dyeing liquid is presented in Fig. 25. Only the control for 10% is presented.

When the bioconversion was carried out in 10 mL glass bioreactors, the change in colour and the appearance of precipitated compounds was visible under the naked eye (Fig. 26).

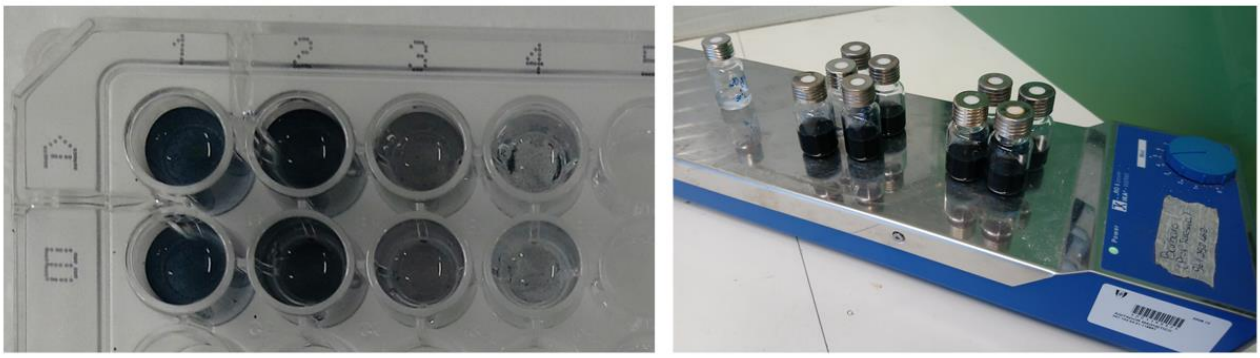

Fig. 24. Biodegradation of the compounds present in Schoeller's dyeing liquid after the dyeing process by Pvec11 in microtitre plate (left) and small-scale reactors (right).

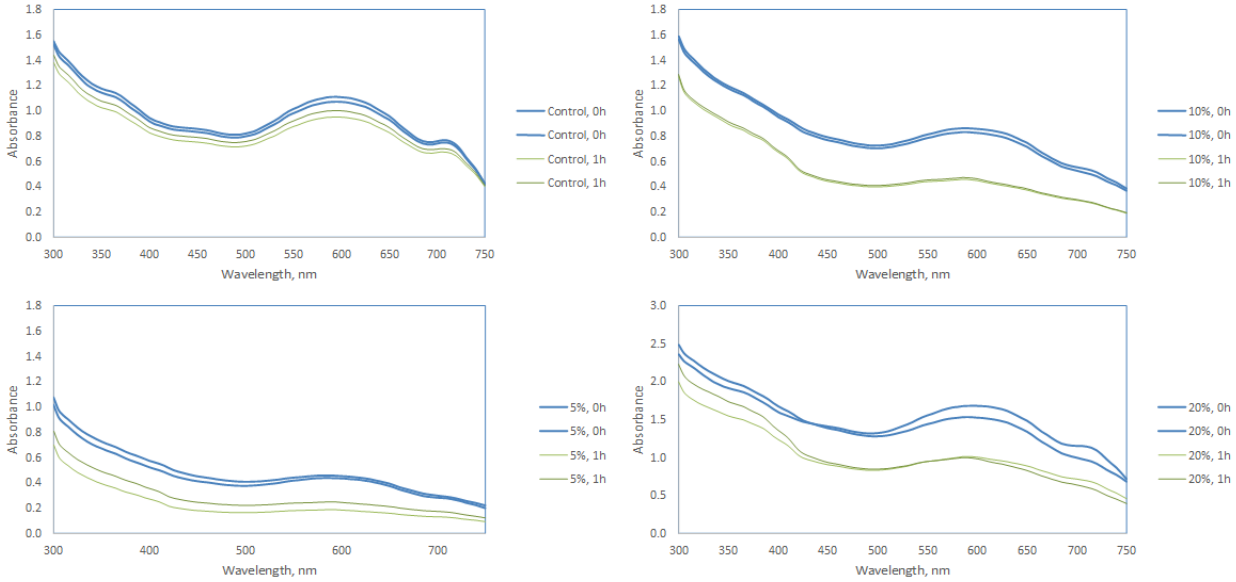

Fig. 25. Spectra of solutions containing 5, 10 or 20% dyeing liquid after the dyeing process at the beginning and after 1h of treatment with Pvec11. Reactions carried out in microtitre plates with 20 mM acetate buffer pH 4.5 containing 1% $CaCl_2$. The control shown contained 10% of dyeing liquid and no enzyme.

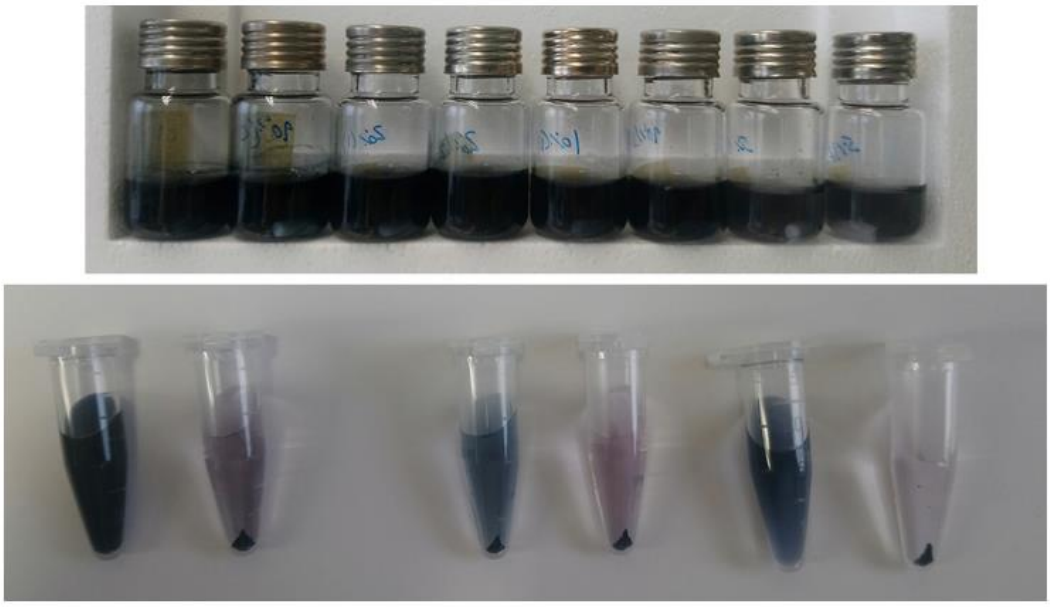

Fig. 26. Biotransformation of dyeing liquid after the dyeing process by Pvec11 in small glass reactors. The appearance of the liquid after 3h of reaction changed significantly (pink liquid) in comparison to the control samples (grey liquid) after centrifugation.

The spectra of each liquid changed significantly during the bioconversion (Fig. 27).

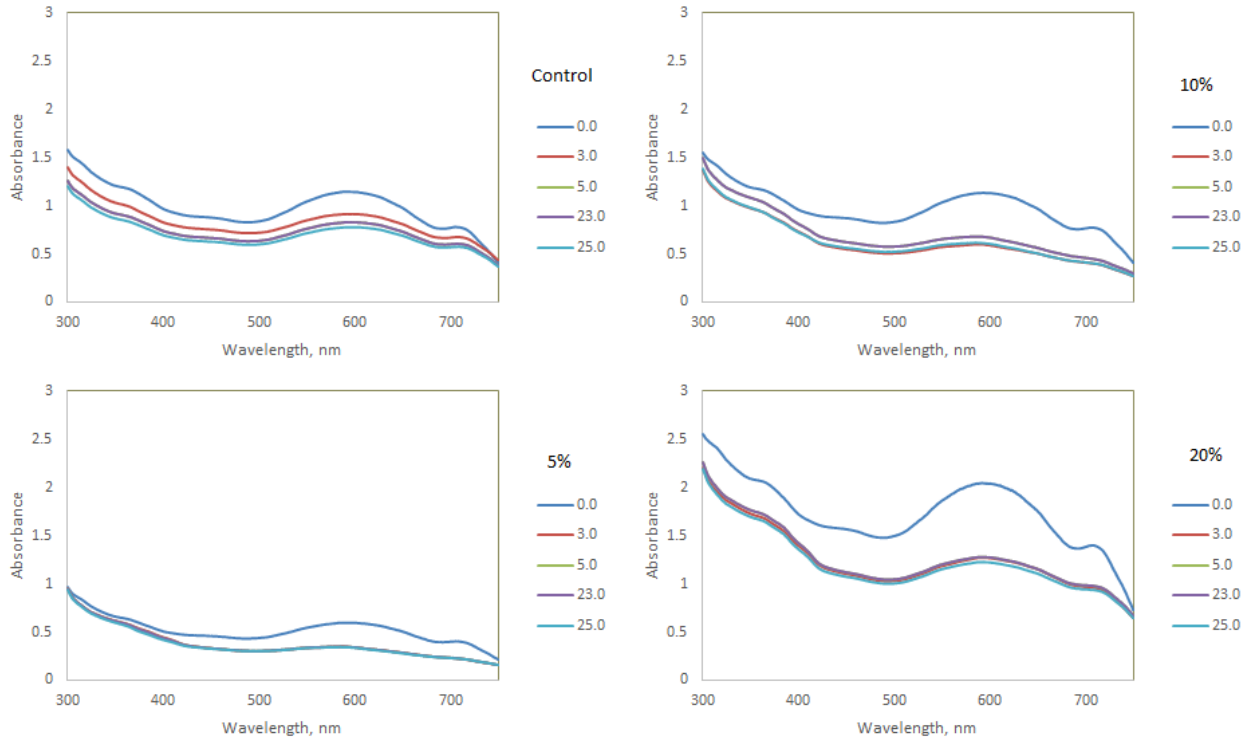

Fig. 27. Spectra of solutions containing 5, 10 or 20% dyeing liquid after the dyeing process at the beginning and after 3, 5, 23 and 25h of treatment with Pvec11. Reactions carried out in small scale reactors with 20 mM acetate buffer pH 4.5 containing 1% $CaCl_2$. The control shown contained 10% of dyeing liquid and no enzyme.

Conclusions sub-section 8 - detergents. The spinning oils from Schoeller fabrics were effectively removed using various enzymes under conditions mimicking industrial treatment, yet none achieved the level of chemical treatment. Enzymes demonstrated effectiveness in degrading fatty acids but fell short of complete removal. Lip9 Val161Ser and bacterial strains also showed promise in removing some compounds but did not match industrial treatment efficacy. Additionally, the enzyme Pvec11 exhibited potential in cleaning dyeing liquid, with visible changes observed in color and appearance during bioconversion in glass bioreactors. Spectral analysis further highlighted significant changes in liquid composition during bioconversion. These findings underscore the potential of enzymes in textile treatment processes but indicate the need for further optimization to match or surpass current industrial standards.

9. Conclusions

In conclusion, based on the results described in this document, we consider that we have achieved the objectives of Deliverable D7.1. In brief, Work Package 7 (WP7) has as objective identify new enzymes for detergent, textile, and cosmetic applications through small to medium-scale trials. In the frame of Deliverables D6.1, D6.2 and the present one (D7.1) a total of 19 enzymes, out of 46 in the pipeline, have been produced at scales from 1 to 176 grams for validation trials and through a series of trials we assessed enzyme performance under various conditions and substrates. The validation trials conducted by both academic and industrial partners contributed to the selection of the most promising enzymes for further development and integration into commercial products. Currently, these candidates include PHE-Paes-PE-H-Y250S, EstLip-PtEst1 and possible Lip9 Val161Ser lipases are the most promising candidates for full-scale wash. Considering the presence of calcium, because its capacity to enhance enzyme activity, should be evaluated. Also, Hyal_HRDSV_2334 hyaluronidase for the production of hydrolytic products meeting Evonik's specifications (within the 1-2 kDa range) using as starting material HA raw 600-900kDa due to its cost advantage according to Evonik's preferences. Finally, the spinning oils from Schoeller fabrics were effectively removed using various enzymes (particularly, Lip9 Val161Ser lipase and some bacterial strains) under conditions mimicking

industrial treatment, yet none achieved the level of chemical treatment. Additionally, the enzyme Pvec11 exhibited potential in cleaning dyeing liquid, with visible changes observed in color and appearance during bioconversion in glass bioreactors, at low temperatures (35ºC). In addition to the testing of enzyme materials in the pipeline (not yet tested or in progress), and optimization loops are proposed for engineering the best candidates, with potential feedback to engineers. A specific timeframe for full-scale validation trials by industry partners will be communicated, with initial trials scheduled after the general meeting in May. Furthermore, while washing trials in textile and detergent applications have been conducted by industrial partners, these have not yet been developed regarding the inclusion of hyaluronic acid degradation products in cosmetics. That being said, ongoing optimization of reaction parameters aims to ensure consistent product quality, with a focus on using HA raw material of 600-900 kDa due to its cost advantage according to Evonik's preferences. Production targets are set at 10-25 g of hydrolysis products, while testing will encompass quality assessment, formulation compatibility, bioactivity, efficacy, and toxicity evaluation. These findings inform our next steps toward delivering hydrolyzed HA products tailored to Evonik's requirements.

10. Final Remarks

In light of the comprehensive findings and detailed analyses presented in this report, it is clear that the objectives of the Deliverable D7.1 outlined at the onset of this project have been successfully met. A copy of the submitted Deliverable D7.1 has been recorded in the intranet's project website. See www.futurenzyme.eu -> login -> private-area -> DELIVERABLES & MILESTONES -> DELIVERABLES -> D7.1_ Report on Small/Medium Validation Trials of 18 Best Pre-Selected Enzymes. Due to its extensive size, the Table S1 with information about the full list of enzyme candidates in the pipeline for production at gram scale are provided in the www.futurenzyme.eu -> login -> private-area -> Shared data -> Datasets -> Table S1 for D7.1_ Report on Small/Medium Validation Trials of 18 Best Pre-Selected Enzymes. Due to its extensive size, the video showing the fabric cleaning process (textile applications) is shown in the www.futurenzyme.eu -> login -> private-area -> Shared Materials -> Videos -> Video S1 for D7.1_ Report on Small/Medium Validation Trials of 18 Best Pre-Selected Enzymes.