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*Call*

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*FuturEnzyme:*

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

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First set of best enzymes

at gram scale

MS19

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## Document information sheet

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## First set of best enzymes at gram scale

## 1. Introduction

Report available – this milestone attests the realisation of the first production batches at gram scale for best enzymes to be used for tests prior to preindustrial validations. Along the already 18 months of the project, different deliverables have been accomplished from which the present milestone nourishes. To be mentioned:

* D5.1\_ The shortlist of at least 18 enzymes nominated for engineering (November 2022)

*This deliverable consists in a report detailing the best 23 enzymes nominated for WP5 (genetic and supramolecular engineering) out of the initial set of enzymes subjected to characterization. The priority candidates for WP6 (large scale production) and WP7 (pre-industrial validations) were also pointed, that are listed in* ***Table 1****.*

**Table 1**. Summary of enzymes and isolates selected as best candidates for WP5-WP7.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Name** | **Partner** | **Priority** | **Signal Peptide** | **Nagoya protocol** | **Application** |
| FE\_Lip9 (lipase) | CSIC | Yes (1st) | Yes | Information provided | Detergent, textile |
| FE\_ID9 (lipase) | CSIC | Yes (1st) | No | Information provided | Detergent |
| FE\_Polur1 (lipase) | CSIC | Yes (2nd) | No | Information provided | Detergent |
| EstLip\_Dim\_#008 (lipase) | UDUS | Yes (1st) | No | Information provided | Detergent |
| EstLip\_Paes\_TB035 (lipase) | UDUS | Yes (2nd) | Yes | Information provided | Detergent |
| EstLip\_PtEst1 (lipase) | UDUS | Yes (2nd) | No | Information provided | Detergent |
| PEH\_Paes\_PE-H\_Y250S (PETase) | UDUS | Yes (1st) | Yes | Information provided | Detergent, textile |
| *V. diabolicus*; *V. alginolyticus* (hyaluronidase) | CNR | Yes | - | Information provided | Cosmetic |

Among the priority candidates, two were selected for first production batches at gram scale, namely, lipases FE\_Lip9 and FE\_ID9. Based on the results gathered by the partners and summarized in the present document, Milestone 19 “First set of best enzymes at gram scale”, can be considered achieved.

## 2. Description of enzymes to be produced at gram scale

As detailed in D5.1 “The shortlist of at least 18 enzymes nominated for engineering” (November 2022), the reasons for selecting FE\_Lip9 and FE\_ID9 lipases as among the best candidates for being produced at gram scale were as follows:

### FE\_Lip9

Activity: Lipase

Partner: CSIC, BSC

Source: MarRef - Marine Metagenomics Database

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

Expression level: Soluble (expression +)

Sequence (signal peptide underlined):

MKVMFVKKRSLQILIALALVIGSMAFIQPKEVKAAEHNPVVMVHGIGGASYNFFSIKSYLATQGWDRNQLYAIDFIDKTGNNRNNGPRLSRFVKDVLDKTGAKKVDIVAHSMGGANTLYYIKNLDGGDKIENVVTIGGANGLVSSRALPGTDPNQKILYTSVYSSADLIVVNSLSRLIGARNVLIHGVGHIGLLTSSQVKGYIKEGLNGGGQNTN

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

MAHHHHHHVGTGSNDDDDKSPDPMAEHNPVVMVHGIGGASYNFFSIKSYLATQGWDRNQLYAIDFIDKTGNNRNNGPRLSRFVKDVLDKTGAKKVDIVAHSMGGANTLYYIKNLDGGDKIENVVTIGGANGLVSSRALPGTDPNQKILYTSVYSSADLIVVNSLSRLIGARNVLIHGVGHIGLLTSSQVKGYIKEGLNGGGQNTN

Biochemical features:

* Most active at temperatures close to 25-40˚C, in agreement with its Td (41.70 ± 1.29°C)
* FE\_Lip9 retains more than 80% of its maximal activity at pH from 7.0 to 10.0.
* It also showed a half life time of 3.5 h in washing liquor.
* FE\_Lip9 was selected based on activity with short to large triglycerides.
* FE\_Lip9 is also capable to degrade all the stained fabrics tested (Pigment with oil on polyester/cotton (PC-09), Mayonnaise on cotton (C-S-05S), Lipstick, pink on polyester/cotton (P-S-16), Fluid make-up on cotton (C-S-17), High discriminative sebum BEY on polyester/cotton (PC-S-132), Beef fat on cotton (C-S-61) and Butterfat on cotton (C-S-10)), showing a preference for Butterfat on cotton (C-S-10).
* We also performed a free fatty acids assay with Schoeller’s textiles where FE\_Lip9 showed a broad capacity to clean the oils in all raw textiles (61488F1, 3X58, 67007, 61988F1, 5237-00 and E03130).
* FE\_Lip9 do show PETase and BHETase activities, being capable of degrading PET.
* The lipase has been successfully subjected to supramolecular engineering (FHNW).
* The enzyme was successfully produced at 10 L scale: 3.96 grams of pure protein obtained.

Comments: this lipase is similar to one patented lipase (US 2009/0325240 A1 or EP 2260105 A2, US 5427936 A and maybe US 2007/0202566 A1). Currently we are using computational and HMM tools to identify sequences with lower level of homology to FE\_Lip9. Also, BSC and CSIC are already selecting and producing a number of mutants to improve the activity towards long oils and the thermo-stability. Until now, two mutants have been designed (Val161Cys and Val161Ser), the synthesis of which is done and expression and activity level will be soon evaluated.

Nominated for: as priority for additive for Henkel detergent and for removal of spinning oils in Schoeller’s fabrics; nominated for WP5 (genetic and supramolecular engineering), WP6 (large scale production) and WP7 (pre-industrial validations).

Nagoya protocol compliance: the sequence was identified by homology screen in the MarRef - Marine Metagenomics Database, a manually curated marine microbial reference genome database that contains completely sequenced genomes (https://mmp2.sfb.uit.no/marref/). This protein is similar to one isolated from a strain isolated from the marine sponge in the seawater in front of Seongsan-ri, Jeju Island, South Korea (33.38°N 126.53°E) (E-value of 8,56E-137 with WP\_034624255.1\_MMP06016472 MULTISPECIES: esterase [Bacillus] [mmp\_id=MMP06016472] [mmp\_db=marref]). Sample was collected 2011-11 (https://www.ebi.ac.uk/biosamples/samples/SAMN06016472), so that before the Nagoya Protocol entered into force on 12 October 2014.

Patent match information (BLASTP against the Patented Protein Sequences Database, only shown those with >95% identity):

* Sequence 2 from patent US 5427936, 99.45% (accession AAA71046.1)
* Lipase [*Bacillus pumilus*], 99.45% (accession CAA02196.1)
* Sequence 1519 from patent US 10676751, 98.90% (accession QPT78109.1)
* Sequence 1537 from patent US 10676751 97.79% (accession QPT78118.1)
* Sequence 26 from patent US 8298799 96.69% (accession AFX20498.1)
* Sequence 73 from patent US 6858422 96.13% (accession AAY02152.1)
* Sequence 1517 from patent US 10676751 95.32% (accession QPT78108.1)
* Sequence 66 from patent US 6858422 95.03% (accession AAY02145.1)

### FE\_ID9

Activity: Lipase

Partner: CSIC

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

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

Expression level: Soluble (expression +)

Sequence (no signal peptide):

MTNLSKPIPNPREYPILPPDMNYIYFENAHLFPFEPEKRDYSPVNAWWLSECAFLVYCHPGFARMAMALVGFDHFHFFQGKGTECMVSWNKDSIIVAFRGTEMKSLSAFHELRTDLNTAPVDFDKGSKVHKGFLKGLQEIWEGEEGLKLFLETLSAEAPSRSMWICGHSLGGALAALCFARLEKASGLYIYGAPRIGDGEFVRICDNRPVWRVEHGRDPIPLVPPDVPALNFNFKDMGKLIYIDYRGEILFERPLVTVEEEKSKVLLNISQQRKRRESLSVEGFKGVLDKDRAKTLINGINEHIMQSRVEWKEYFDSLDKGIGLKIKDHMPIYYCAKLWNILIEGL

Synthesized sequence once introduced in the expression vector:

MAHHHHHHVGTGSNDDDDKSPDPMTNLSKPIPNPREYPILPPDMNYIYFENAHLFPFEPEKRDYSPVNAWWLSECAFLVYCHPGFARMAMALVGFDHFHFFQGKGTECMVSWNKDSIIVAFRGTEMKSLSAFHELRTDLNTAPVDFDKGSKVHKGFLKGLQEIWEGEEGLKLFLETLSAEAPSRSMWICGHSLGGALAALCFARLEKASGLYIYGAPRIGDGEFVRICDNRPVWRVEHGRDPIPLVPPDVPALNFNFKDMGKLIYIDYRGEILFERPLVTVEEEKSKVLLNISQQRKRRESLSVEGFKGVLDKDRAKTLINGINEHIMQSRVEWKEYFDSLDKGIGLKIKDHMPIYYCAKLWNILIEGL

Biochemical features:

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

Nominated for: as priority for additive for Henkel detergent; nominated for WP5 (genetic and supramolecular engineering), WP6 (large scale production) and WP7 (pre-industrial validations).

Nagoya protocol compliance: ID9 (orignal name EstLip\_NODE\_494\_length\_56501\_cov\_3.272419\_27) was isolated from the microbial assemblages from bone surface and the bone-eating worm *Osedax mucofloris* (BioProject ID PRJNA606180), collected at Byfjorden, Bergen, Norway (60.397093N; 5.301293E). The samples were collected from 01.2017 to 11.12.2017 in Norway, in the frame of the EraNet project ProBone. Norway was among the first when ratifying the Nagoya protocol so that Nagoya protocol applies; all documentation (including pictures) that shows where the samples were taken can be made available upon request.

Patent match information (BLASTP against the Patented Protein Sequences Database, only shown those with >95.5% identity):

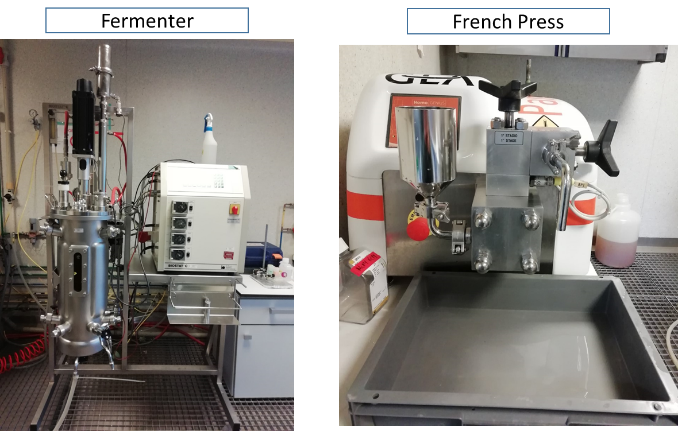
* Sequence 7 from patent US 9527906, 100.00% (accession ATJ64490.1)
* Sequence 62 from patent US 9884101, 100.00% (accession AVY22639.1)

## 3. Materials and equipment

Materials. His6-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 NaH2PO4, 236.75 mL 0.2 M Na2HPO4, 100 mL 3 M NaCl, 10 mL 1 M imidazol and 640 mL H20. The elution buffer (50 mM sodium phosphate buffer pH 8.0) was prepared by mixing 6.625 mL 0.2 M NaH2PO4, 118.375 mL 0.2 M Na2HPO4, 50 mL 3 M NaCl, 125 mL 1 M imidazol and 200 mL H2O.

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.

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**Figure 1**. 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.

## 4. Methods

**Fermentation at milligram scale**

The FE\_Lip9 and FE\_ID9 His6-tag enzymes are available in pET-45b(+) and the host *E. coli* BL21(DE3).

* One colony is picked and used to inoculate 50 mL of Luria Bertani (LB)1 broth plus antibiotic (ampicillin [Amp] 50 µg/mL) in a 0.1-0.25-L flask.
* The cultures were then incubated at 37°C and 150 rpm until an OD between 0.8 - 1 (OD is measured at 600 nm).
* The next day, add this pre-inoculum to a 2.5 L-flask with 1 liter of LB + Amp 50 µg/mL and let it grow at 37°C and 150 rpm until an OD between 0.8 - 1 (OD is measured at 600 nm).
* Induce the culture.
  + The plasmid is pET45b(+) is used, which requires 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) for induction.
* Leave the culture inducing overnight at 16°C2 and 200 rpm.
* Centrifuge the cultures at 8000 g for 30 minutes at 4°C. *Note*: the centrifugation speed and the temperature can be adapted as the enzymes are stable at room temperature.
* The pellet is resuspended in 20 mL of 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.0 to wash the pellet, centrifuged at 8000 rpm 5 minutes and at 4°C. *Note*: the buffer can be adapted as needed (for example phosphate buffer, Tris buffer, etc.), but we recommend keeping neutral pH (7.0-7.5).
* The pellet is resuspended in 20 mL of 40 mM HEPES pH 7.0. Then, Lysonase Bioprocessing reagent is added (4 μL g–1 wet cells) and incubated for 60 min on ice with rotating mixing. *Note*: instead of Lysonase Bioprocessing reagent, lysozyme can be added (1 mg/mL).
* The cell suspension was sonicated using a pin Sonicator 3000 (Misonix, New Highway Farmingdale, NY, USA) for a total time of 5 min (10 W) on ice and centrifuged at 15000 *g* for 15 min at 4°C. *Note*: instead of sonication, cell lysis by other methods such as French Press can be used.
* The supernatant was retained and directly used for protein purification.

**Fermentation at gram scale**

The FE\_Lip9 and FE\_ID9 His6-tag enzymes are available in pET-45b(+) and the host *E. coli* BL21(DE3).

1. One colony is picked and used to inoculate 80 mL of Luria Bertani (LB)1 broth plus antibiotic (ampicillin [Amp] 50 µg/mL).
2. The cultures were then incubated at 37°C overnight; the final OD is about 2.2 (OD is measured at 600 nm).
3. 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).
4. Leave the culture overnight.
5. Centrifuge the cultures.
6. The pellet (about 69 grams) is resuspended in 286 mL of 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.0.
7. Cells are lysed by French Press (**Figure 1**), after which the suspension was centrifuged at 11000 rpm, 20 min, 4°C.
8. The supernatant was retained and directly used for protein purification.

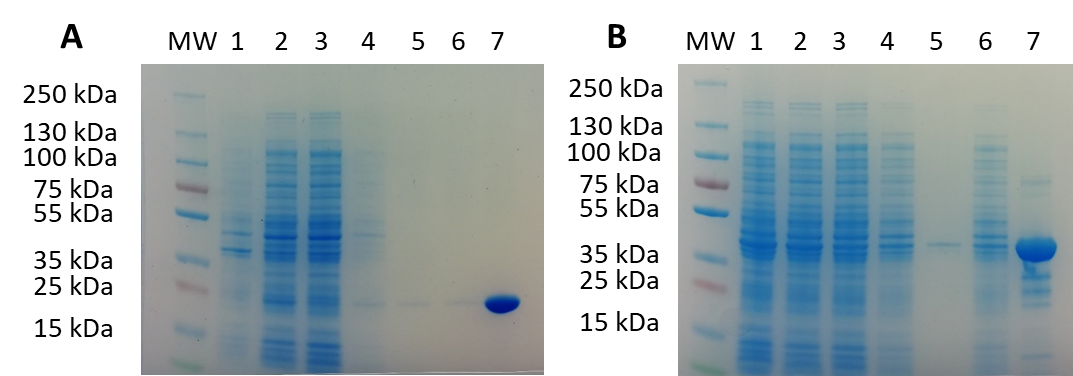
**Purification.** The His-tagged lipases were purified at room temperature after binding to a Ni-NTA His-Bind resin (Sigma Chemical Co. (St. Louis, MO, USA)). Briefly, the supernatant (50 mL) obtained following the steps detailed in the “fermentation sub-section” were mixed with an equal volume of binding buffer (50 mL). A total of 6 mL Ni-NTA His-Bind resin were added to the protein solution and the suspension maintained for one hour under gentle agitation. After incubation, the resin was separated by filtration through a 47 mm diameter glass microfibre filter (MFV1, Filter-LAB). The resin was extensively washed with washing buffer, after which the 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-LyzerTM 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”.

## 5. Results

The production of FE\_Lip9 and FE\_ID9 His6-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 His6-tag lipases, respectively, were obtained from 1-L culture.

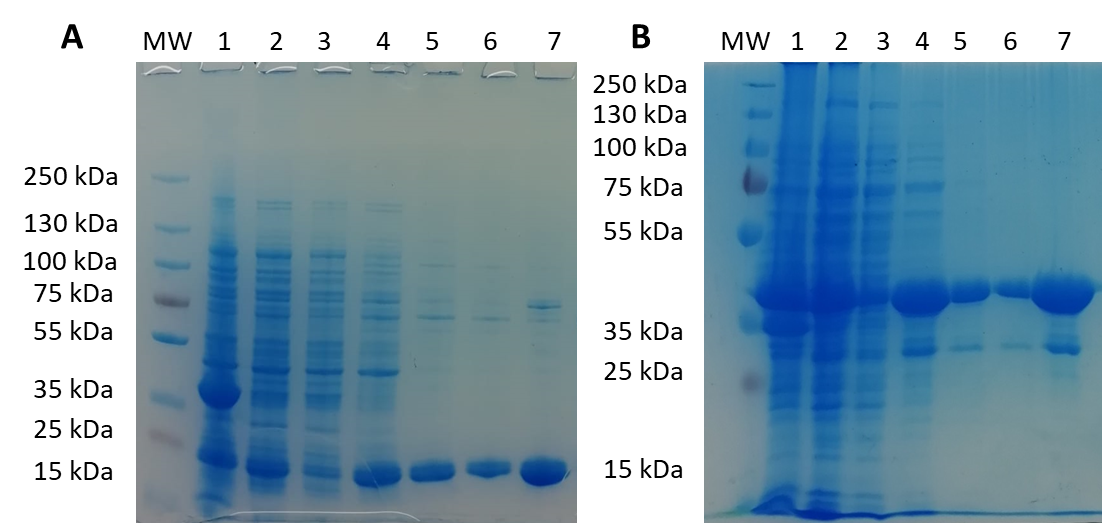
As detailed in the section 2 and also in D5.1 “The shortlist of at least 18 enzymes nominated for engineering” (November 2022), the pure lipase preparations prepared at milligram scale did show activities of interests and for this reason their production at gram scale was undertaken. Following the protocol detailed in section 3, 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 His6-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) His6-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 His6-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 His6-tag lipases purified at gram scale were transferred to partners FHWM and INOFEA (on 23 November 2022) for their supramolecular engineering.