Transition towards environment friendly consumer products by co-creation of an oxidoreductase foundry

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D4.1. Host and expression conditions per enzyme type

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Lead beneficiary-organisation	LEITAT
Responsible person	Aroa Rey Campa (LEITAT)
Name and affiliation contributing persons	L. Santema (RUG), R. Ree (NORCE), A. Garcia-Moyano (NORCE), Ø. Larsen (NORCE), G. Bjerga (NORCE), M. Guillén (UAB), A. Bassegoda (LEITAT)
Name and affiliation internal reviewers	N. Loncar (GECCO), M. Fraaije (RUG), G. Bjerga (NORCE)

Document history and changes

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Contents

1. Summary

OXIPRO aims to generate an oxidoreductase foundry to develop more environmentally friendly consumer products by collaboration of an interdisciplinary team of researchers and industry partners. Oxidoreductases are a type of enzymes capable of replacing harmful and toxic chemicals as well as transform products that have been vastly underused. WP4 focuses on the efficient production of the different oxidoreductases required for the diverse applications on WP5, as well as for the new toolboxes in WP3, such as encapsulation strategies, co-factor regeneration approaches and screening platforms.

The main parameters when developing an efficient recombinant protein expression are cell growth and expression levels. While bacteria are the preferred host for recombinant expression due to its rapid growth in inexpensive culture medium, and the well-known metabolism with many molecular tools for genetic manipulation, not all enzymes are efficiently expressed in bacterial hosts and other alternatives needs to be explored, such as yeast *(Pichia)* or filamentous fungus *(Aspergillus)*. In this Deliverable, work is focusing on *Escherichia coli (E. coli)* as an expression host; however, other expression hosts, such as *Bacillus subtilis (B. subtilis)* and *Pichia pastoris (P. pastoris)*, are being explored and developed in parallel. Here we show some of the different cloning strategies performed to express our oxidoreductase genes of interest, explain the vector choices according to the needs, explore the different growth medium and induction conditions and the purification methods carried out to obtain the enzymes required. Although activity assays have been performed for all the enzymes expressed in this project, description of them are not included in this document, as it falls beyond the scope of this Deliverable.

2. Oxidoreductases

In the following section, a general description of the different enzyme types and their application in this project will be included, as well as reported or public information on cloning strategies, expression hosts and enzyme purification options.

2.1 Alcohol oxidases – hygienizing detergents (LEITAT, RUG, BSC)

Alcohol oxidases (AOX, EC 1.1.3.13) are enzymes that catalyze the oxidation of a primary alcohol into an aldehyde in the presence of molecular oxygen, O_2, releasing hydrogen peroxide, H_2O_2. Other types of alcohol oxidases are known, such as the enzymes capable of oxidizing secondary alcohol (SAO, EC 1.1.3.18), which act in the same way as AOX but with the generation of a ketone and hydrogen peroxide as products. In both cases, the cofactor FAD is required as an electron carrier (Goswami *et al*., 2013).

Laundry detergents are cleaning agents used to remove dirt from clothes. Mainly two types of detergents are available in the market: liquid and solid (Bajpai *et al*., 2007). Solid (powder) detergents include a molecule able to produce hydrogen peroxide when in contact with water, but stable while stored. However, liquid detergents have not been able to incorporate a bleaching agent with the same whitening and disinfectant capability, as hydrogen peroxide is an extremely unstable molecule that cannot be stored in solution (Bockmühl, 2017). The use of alcohol oxidases as an ingredient in laundry detergents allows the production of hydrogen peroxide *in situ*, thereby generating a whitening and disinfectant agent during the washing.

2.1.1 Cloning, Transformation, Mutagenesis and Expression

Synthetic genes encoding *Aspergillus japonicus* Alcohol oxidase (*Aj* AOx) and *Thermopolyspora flexuosa* Alditol oxidase (*Tf* AldO), codon-optimized for *E. coli* with BsaI sites at the 5'and 3' termini were ordered from Twist Biosciences. Both were cloned into a pBAD-His-SUMO vector using the Golden Gate assembly method (Engler *et al*., 2013), which adds a His-tag end to allow easy purification. For transformation, 2 µL of PCR product were mixed with 50 µL of chemically (CaCl) competent *E. coli* NEB DH10β cells and incubated on ice for 30 min. The cells were heat-shocked in a 42^{o}C water bath for 45 s and cooled on ice again for 5 min. 250 µL LB medium was added and the cells were incubated at 37^{o}C, 135 rpm for 1 hour; after which 50 µL of the recovered cells were plated on LB-agar with 50 µg/mL ampicillin and incubated overnight (~16 hours) at 37^{o}C. Cloning was verified through plasmid isolation and Sanger sequencing.

For expression a pre-inoculum in 5 mL LB-amp (50 µg/mL) of the desired *E. coli* NEB DH10β strain was grown overnight at 37°C, 135 rpm, which was used to inoculate a 2 L non-baffled flask containing 400 mL of Terrific Broth (1.2% tryptone, 2.4% yeast extract, 0.5% glycerol in phosphate buffer) supplemented with 50 µg/mL ampicillin. The flask was incubated at 37°C, 135 rpm until an OD_{600} of ~0.5-0.6 was reached. Expression was induced with a final concentration of 0.02% L-arabinose after which; the cultures were incubated at 37°C for ~16 hours. Cells were harvested by centrifugation (6000 rpm, 15 minutes, 4°C) and stored at -80°C.

2.1.2 Protein Purification

Cell pellets were resuspended in buffer A (50 mM KPi and 150 mM NaCl (pH 7.5)) with a 1:10 induction volume ratio (100 mL expression volume results in 10 mL buffer A). Cells were disrupted by sonication (5 s on, 7 s off, 70% amplitude for a total of 15 min) and then centrifuged at 13000 rpm for 50 min at 4°C. The resulting supernatant was loaded on a gravity column containing 4 mL Ni Sepharose previously equilibrated with buffer A to purify the His-tagged protein. Then a washing step was performed with 3 column volumes (CV) of buffer A and 3 CV of buffer B (50 mM KPi, 150 mM NaCl and 20 mM imidazole (pH 7.5)). Proteins were eluted with 3 mL elution buffer (50 mM KPi, 150 mM NaCl and 500 mM imidazole (pH 7.5)), before the buffer was exchanged with storage buffer (50 mM KPi (pH 7.5)) using a PD10 desalting column. The protein obtained can be visualized in the gel (**Figure 4.1.1**).

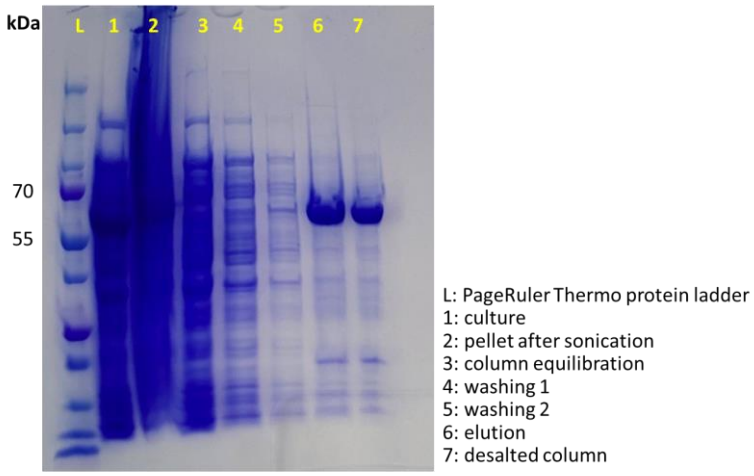

***Figure 4.1.1**. SDS-PAGE AOXTf – samples from the different purification steps. Protein can be visualized at around 60 kDa, which is the expected size.*

To determine the concentration of the purified fusion protein, either Bradford protein assay was carried out, or extinction coefficients were used which were found by SDS unfolding for protein. The yield for TfAldOX was found to be around 250 mg/L. A coupled enzyme test with TfAldOX with glycerol

and HRP (horseradish peroxidase) with ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), a colored molecule that allows spectrophotometric detection, was used to determine the formation of H_2O_2.

While TfAldO was found to be active after expression and purification under the conditions mentioned above, both AjAOX and ChAOX were found to be partly inactive due to the presence of their flavin co-factor in a semiquinone state. In this state, the enzyme is catalytically dead and the only way to reactivate this part is by replacing the co-factor during the purification by unfolding and refolding, which results in a great loss of yield. Several cloning and expression tests were done in different *E. coli* strains (BL21, NEB10 and Rosetta) but no fully active form of these enzymes was reached.

2.2 Carbohydrate oxidases – biobleaching (UAB, RUG)

Carbohydrate oxidases (EC 1.1.3) are oxidoreductases that catalyze the oxidation of the C-OH bond in the carbohydrate molecule, turning them into C=O and releasing hydrogen peroxide. This reaction requires a redox cofactor and molecular oxygen, O_2.

The bleaching process is one of the final steps of cotton processing. Today, chemical treatment by caustic soda and H_2O_2 is used to whiten the fabric. Chemical bleaching occurs at pH 10-12 and high temperatures (90-100°C). Biobleaching of cotton using H_2O_2 generated by glucose oxidase (GOX; EC 1.1.3.4) is a promising solution. The desizing and bioscouring water baths from previous steps of the cotton processing are rich in glucose and other carbohydrates, which can be reused for biobleaching, offering a recycling of the wastewater. Reuse of sugars, albeit not limited to glucose, by a wide selection of carbohydrate oxidases active on these sugars generated from amylase treatment, will be explored.

2.2.1 Host selection and cloning

The selected strain for carbohydrate oxidase production is *E. coli* M15ΔglyA. The deleted gene *glyA* encodes for serine hydroxymethyl transferase (SHMT) enzyme, which catalyses reversible interconversion between serine and glycine. Deletion of *glyA* causes an auxotrophy and makes the strain threonine dependent. Potentially, this characteristic of the strain would allow selection by auxotrophy. The vector used is a ColE1 plasmid derived from pQE-40 vector (Qiagen), called pVEF. Recombinant intracellular protein expression is mediated by IPTG induction and regulated by T5 promoter with double lacO repression module for tightly-regulation repression. Moreover, this vector encodes a β-lactamase for ampicillin-resistance and a *glyA* gene. In addition, a plasmid with no antibiotic resistance is also available in the research group. During the first cultures for production optimization, the expression system harbouring both auxotrophy and antibiotic resistance will be used to facilitate possible genetic modification that could be required to get the final optimum plasmid in terms of productivity maximization. Finally, the plasmid with no antibiotic gene resistance will be used for production up-scaling.

According to the results of experiments carried out within WP5, a carbohydrate oxidase from the University of Groningen was the best candidate to develop the biobleaching process. Therefore, this biocatalyst was the selected enzyme to be produced in WP4. The gene was provided by RUG in a pBAD vector. Enzyme was cloned into pVEF vector to produce the enzyme in *E. coli* M15ΔglyA pVEF system. This system has been developed in-house and fermentation strategies have been characterized for a

wide range of enzymes. SLIC (Sequence and Ligation-Independent Cloning) method was the applied cloning technique which consists of reassembling linear DNA fragments based on the PCR-generated homologous regions at both ends. DNA is treated with T4 DNA polymerase in the absence of oligonucleotides which activates its exonuclease activity. Exonuclease activity generates single-stranded ends which can hybridize due to homology regions. After transformation and selection in LB agar plates (1% tryptone, 0.5% yeast extract, 1% sodium chloride, 1.5% agar) supplemented with 100 µg/mL ampicillin, 11 colonies were selected for further insert validations. For the first validation a PCR and SDS-PAGE gel is performed, expecting to observe a band of around 2200 bp in those colonies which have insert correctly cloned into vector. From these 12 colonies, four were discarded because of either absence of any band or smaller band than expected. After PCR results, a restriction enzyme digestion profile with KspAI is done with those colonies that show an expected size band. KspAI restriction enzyme is selected because there are two restriction sites within the sequence of the gene. All the colonies tested presented the expected restriction profiles. Sequencing confirmed that gene was correctly inserted into the vector which was also validated in previous restriction enzyme digestion profile, but also, that the sequence of the enzyme has no mutations in any of the colonies.

2.2.2 Enzyme production

Enzyme production tests have been performed in shake flask scale with Terrific Broth (TB) medium as well as in a bioreactor with defined medium Firstly, a test to determine the best temperature for induction was carried out in 25 mL shake flasks using TB medium. Enzyme expression was tested at induction temperatures of 25°C and 30°C. Preinduction culture growth in Terrific Broth complex medium was performed at 37°C. Induction phase started when preinduction culture was inoculated to a fresh TB + 200mM IPTG medium at 25°C and 30°C. Initial OD in both tests was 0.6. At both temperatures an OD value between 5.5 – 6.0 was reached at the stationary phase. Induction phase at 25°C lasts 13 h, 10 h in the case of 30°C. After disruption, insoluble (pellet) and soluble (supernatant) fractions were separated by centrifugation at 10000 *g* for 14 minutes at 4°C. Both fractions from cultures at 25°C and 30°C were analysed by SDS-PAGE.

The results confirmed the expression of the recombinant protein. Regarding the level of expression observed in each case, there was no great difference between the two temperatures tested neither in the soluble nor the insoluble fraction.

The catalytic activity was quantified by measuring the hydrogen peroxide (H_2O_2) formation by an enzymatic coupled reaction with horseradish peroxidase (HRP) and two chromogenic peroxidase substrates: 4-aminoantipyrine (APP) and 3,5-dichloro-2-hydroxybenzenesulfonic (DCHBS).

Activity of soluble fraction was higher than that of insoluble fraction in all clones with no significant differences between both induction temperatures. These results were as expected since the lack of solubility of the protein could be due to a misfolding, thus leading to a decrease of enzyme activity. In soluble fraction, the activity of 25°C induced-culture doubles activity of 30°C induced-culture due to a better folding of the protein thanks to a decrease in the enzyme production rate cause by the lower temperature. Solubilization strategies by refolding of the protein present in the insoluble fraction was not considered at this point but trying to increase the enzyme correctly folded by means of bioprocess optimization (Task 4.2).

Production in complex medium was also scaled up to 1 L flask to produce enzyme to continue WP5 tasks while the optimization of production using defined medium is ongoing. In this case initial OD was 0.3 and culture was stopped at 6 OD after 16 h induction. Sample processing and purification tests are described in section 2.2.3.

The next step in optimizing the production process was switching to a defined medium. Generally, defined media have a lower cost than complex media, which is relevant in light of upscaling costs. In addition, defined media is of known composition while complex media are heterogeneous which reduces process reproducibility. Also, in defined media a limiting substrate (e.g., glucose) can be used to increase process control in fed batch strategies.

Production in defined medium was made directly in 2 L bioreactor. Initial OD was 0.2 and the culture grew up for 13h before induction with 0.2 mM IPTG (OD 5.2). Induction continued during 9h until growth rate slowed down. Activity on the lysate showed that it kept increasing although cell growth stopped. Maximum activity measured in the lysate was 0.57 AU/mg DCW.

Enzyme production in defined medium has been significantly lower than in TB medium, although the fermentation that was carried out in bioreactor can be redesigned and optimized (moment of induction, time of harvest, etc.) for better performance according to the results obtained. This

optimisation will be carried out in further steps, for production of the enzyme with CBM-tag, implementing a fed-batch strategy to increase enzyme expression productivity.

2.2.3 Purification tests

The fermentation broth with a final OD of 6 was centrifuged at 6000 rpm, for 20 min at 4°C to recovery the biomass. The recovered biomass was storage at -20°C. 29.8 g of wet biomass was resuspended in 15 mL of buffer A (500 mM NaCl, 100 mM KH_2PO_4, pH 7.5), reaching an OD of 140. The sample was then disrupted using a OneShot disruptor (Constant Systems), for 2 cycles at 1.72 Kbar. The lysate was centrifuged at 10000 x *g* for 15 min at 4°C, to remove the cell debris and recovery 13 mL of lysate with 209.2 AU/mL of oxidative activity.

The enzyme was purified using fast protein liquid chromatography (FPLC) in an ÄKTA Pure (GE Healthcare®, Chicago, IL, USA), with a column of Sepharose-Ni^{+2}.

Even though the target enzyme could be detected in the not retained fraction as well as in the wash, it was mainly recovered in the elution fraction. After the purification process, 4.5 mL solution of purified enzyme were obtained with 217.1 AU/mL, which corresponds to 93% of the total activity loaded in the process.

2.3 Trimethylamine monooxygenases – clinical nutraceuticals (NORCE, BSC)

Flavin containing monooxygenases (FMOs, EC 1.14.13.8) are enzymes that insert one molecule of oxygen into organic substrates using the cofactors FAD and NAD(P)H (Ceccoli et al., 2014; Torres Pazmiño et al., 2010; van Berkel et al., 2006). A subgroup of bacterial FMOs oxidize trimethylamine (TMA) to trimethylamine N-oxide (TMAO) and are often referred to as trimethylamine monooxygenases (Tmms) (Chen et al., 2011; Choi et al., 2003; Goris et al., 2020). TMA is a well-known contributor to the odor of spoiled fish (Hebard et al., 1982).

Application of Tmm enzymes is an alternative and novel strategy to convert TMA to the odorless TMAO in fish protein hydrolysates (Goris et al., 2020). This strategy has the potential to significantly improve the organoleptic quality of fish protein hydrolysates and thereby promoting their application as food ingredients, while simultaneously maintaining their nutritional profile.

Using a specific bacterial Tmm as a starting point, OXIPRO is developing variants by computational approaches to align their performance with process parameters. OXIPRO will also be looking into naturally occurring variants of Tmms to gain a broader understanding of their functionalities.

2.3.1 Molecular cloning of Tmm wild-type and variants

The native Tmm genes and mutant variants were commonly ordered as synthetic genes, codon-optimized for expression in the relevant host, such as *E. coli*. The genes were then typically subcloned into the expression vector pBXC3H (p12) by fragment exchange (FX) cloning to add C-terminal His-tags to the encoded gene, as previously described (Bjerga et al., 2016; Geertsma and Dutzler, 2011; Goris et al., 2020). Briefly, subcloning was performed using the *E. coli* MC1061 strain and LB-agar (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 1% [wt/vol] NaCl, 1.5% [wt/vol] agar-agar) supplemented with ampicillin (100 µg/mL, Sigma-Aldrich) for selection. The resulting plasmids, purified using the NucleoSpin plasmid kit (Macherey-Nagel, Germany), were confirmed by Sanger sequencing.

2.3.2 Tmm protein expression and purification

At small-scale for initial screening of enzyme activity, enzymes were expressed in *E. coli* MC1061 cells in 24-well deep-well plates in LB medium (Sigma-Aldrich) supplemented with 0.1% (w/v) L-tryptophan (Sigma-Aldrich) and 100 µg/mL ampicillin at both 20°C and 30°C for 16 h after induction with L-arabinose (Sigma-Aldrich) to a final concentration of 0.1% (w/v). Proteins were purified using Ni-NTA spin columns (Qiagen), following the Qiagen protocol. Cells were collected by spinning at 4,000 × *g* for 30 min at 4°C and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM

phenylmethanesulfonyl fluoride [Sigma-Aldrich], 0.1% *n*-dodecyl β-d-maltoside [Sigma-Aldrich], 5 µg/mL DNase [Sigma-Aldrich], 0.2 mg/mL lysozyme [Sigma-Aldrich]). Columns were equilibrated with equilibration buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM imidazole) before incubation with cell lysate and washed with wash buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 30 mM imidazole). Proteins were eluted with elution buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 500 mM imidazole). Total protein fractions of cell lysate, soluble fractions, and eluted proteins were analyzed by SDS-PAGE (Bio-Rad). Activity assays were used to confirm the presence of functional enzyme.

At medium-scale, native Tmm and mutant variants were expressed in *E. coli* MC1061 cells in 100 mL LB-medium supplemented 100 µg/mL ampicillin at 20°C for 16 hours after induction with 0.1% (w/v) L-arabinose. All purification steps were conducted on ice or using ice cold buffers. Cells were harvested by centrifugation, resuspended in lysis buffer, lysed by freeze thaw cycles and sonication, and cleared by centrifugation. The His-tagged Tmm enzymes were then purified from the cleared lysate using Ni-NTA resin. The buffer of the eluted protein was changed to 50 mM Tris-HCl, pH 7.5, 100 mM NaCl using PD10 columns (GE Healthcare). Finally, the protein was concentrated using protein concentrator columns (Thermo Fisher) and stored with 10% glycerol at -20°C until further use. Protein concentrations were measured using the PierceTM 660 nm Protein Assay Reagent (Thermo Fisher) with BSA as standard, and purity was assessed by SDS-PAGE. Activity assays were used to confirm the presence of functional enzyme (data not presented, as activity assessments are outside the scope of this Deliverable).

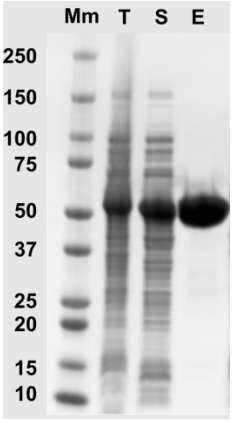

Figure 4.1.2. *Expression and purification a Tmm enzyme. The enzyme was expressed in E. coli and purified by Ni-NTA affinity chromatography. The figure shows an SDS-PAGE gels documenting the total protein (T) after expression, the soluble protein (S) present in the clarified lysate, and the eluate after Ni-NTA purification (E). Molecular masses (kDa) of the protein standard molecular marker (Mm) are shown to the left.*

2.4 Dehydrogenases for NADH/NADPH cofactor regeneration (NORCE)

Structurally, class B flavin-dependent monooxygenases, such as flavin-containing monooxygenases (FMOs), contain two Rossmann fold domains that harbor dinucleotide binding motifs, namely, for their

tightly bound flavin adenine dinucleotide (FAD) and nicotinamide dinucleotides (NADH/NADPH) cofactors, on which the enzymes depend for their catalytic action. The availability of the cofactor is important to consider for the implementation of an enzyme in an industrial process. In OXIPRO, several strategies are undertaken to advance cofactor dependence, one of these includes using accessory or secondary enzymes to regenerate the cofactor. Dehydrogenases are relevant in this context.

2.4.1 The application of dehydrogenases in the OXIPRO innovation case of nutraceuticals

In a proof-of-concept experiment performed on a salmon hydrolysate, Tmm enzymatic activity against TMA was only observed after the cofactor NADPH was added to the hydrolysates (Goris et al., 2020). Cofactor dependency of Tmm enzymes must therefore be addressed. NADPH is an expensive chemical, and hence, adding NADPH to an industrial process is highly unlikely to be cost effective. One solution is to engineer the enzymes to accept alternative cofactors (Chanique et al., 2018), which may be present in the TMA-rich biomaterial of interest or are less expensive to add. Another solution is to regenerate the cofactor (Hummel and Groger, 2014; Aalbers and Fraaije, 2019). Flavin binding monooxygenases, such as class I type I BVMOs, have been fused to a glucose dehydrogenase to obtain a continuous oxidation reaction driven by the dehydrogenase-catalyzed regeneration of NADPH from $NADP^+$ (Mourelle-Insua et al., 2019).

One of the two approaches undertaken in OXIPRO involves the use of enzyme cascades by co-expressing enzymes or making them fusion partners for the regeneration of the nicotinamide cofactor. Effective cofactor regeneration requires a second coupled enzyme. Both glycerol, glucose and formate dehydrogenase enzymes have been successfully coupled to other monooxygenases. These coupled enzymes use sacrificial co-substrates that are inexpensive and readily available. OXIPRO will explore the use of a regeneration enzyme which is compatible with the final hydrolysate food-grade formulation. Dehydrogenases identified in T.2.3. and produced in T.4.1. will herein be assessed in colorimetric assays. The optimal enzyme will be combined with the model Tmm enzyme to set up a continuous NAD(P)H assay reaction. The two combined approaches will allow the full exploitation of the pool of redox cofactors in the marine biomass, bypassing the need for any external addition.

2.4.2 Molecular cloning of G6PDH

As a model enzyme, the gene encoding glucose-6-phosphate dehydrogenase (G6PDH) from *E. coli* was PCR amplified from *E. coli* MC1061 using the primer set 5'-ATATATGCTCTTCTAGTGCAGTAACGCAAACAGCCCAGGCCTGT and 5'-

TATATAGCTCTTCATGCCTCAAACTCATTCCAGGAACGACCATC before cloning by FX-cloning into vector pINITIAL (Geertsma and Dutzler, 2011). This construct was used as template for further subcloning into the pBXNH3 (p1) by FX-cloning to generate an N-terminal His-tag construct. The constructs was not verified by sequencing.

2.4.3 Expression and purification of G6PDH

G6PDH was recombinantly overexpressed in *E. coli* MC1061 in 200 ml 2xYT medium (1.6% [w/v] tryptone, 1.0% [w/v] yeast extract, and 0.5% [w/v]) supplemented with 0.1 mg/mL ampicillin at 20°C for 24 hours after induction with 0.1% L-arabinose. Cells were harvested by centrifugation at 4°C before resuspended in lysis buffer (50 mM TrisCl. pH 7.5, 50 mM NaCl, 1 mM phenylmethanesulfonyl fluoride [Sigma-Aldrich], 0.3 mg/mL lysozyme [Sigma-Aldrich]) The cells were broken by sonication in an ice/water batch and cleared by centrifugation. The His-tagged G6PDH enzyme was then purified from the cleared lysate using Ni-NTA resin (Qiagen). The buffer of the eluted protein was changed to 50 mM Tris-HCl, pH 7.5, 100 mM NaCl using PD10 columns and stored with 10% glycerol at -20°C until further use. Protein concentrations were measured using the PierceTM 660 nm Protein Assay Reagent (Thermo Fisher) with BSA as standard, and purity was assessed by SDS-PAGE. Activity assays were used to confirm the presence of functional enzyme.

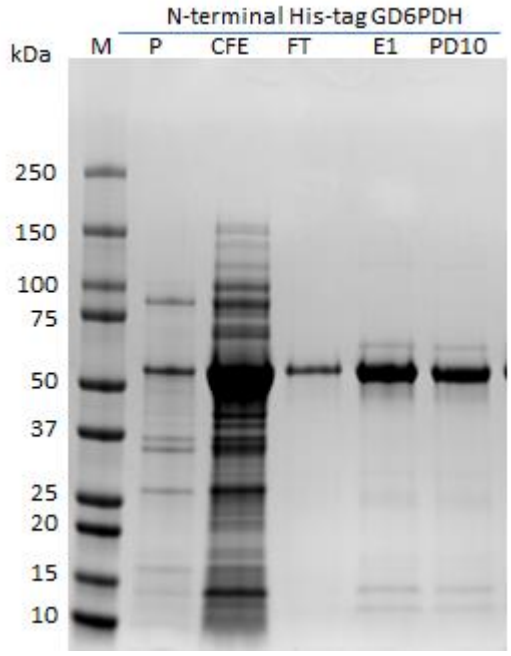

***Figure 4.1.3**. Expression and purification of N-terminal His-tagged glucose-6-phosphate dehydrogenase (G6PDH) from E. coli. The enzyme was expressed in E. coli MC1061 and purified by Ni-NTA affinity chromatography. The figure shows SDS-PAGE gels documenting the insoluble proteins (P) after expression, the soluble proteins (CFE) present in the clarified lysate, the flow-through (FT) from the Ni-NTA column, the eluate after Ni-NTA purification (E1), and the desalted enzyme preparation from the PD10 column. Molecular masses (kDa) of the protein standard molecular marker (M) are shown to the left of the gel.*

2.5 Laccases – polymers with UV absorption (LEITAT)

Laccases (EC 1.10.3.2) are multi-copper oxidases that catalyze the oxidation of a wide variety of phenolic compounds leading to crosslinking. They react in the presence of molecular oxygen, O_2, releasing H_2O and are therefore considered ecofriendly (Arregui *et al*., 2019).

In OXIPRO, laccases are applied in the cosmetic case study for the biocatalytic synthesis of melanin-like polymers with ultraviolet (UV) absorption properties as sun care ingredients with minimal impact on the aquatic life. Different phenolic and polyphenolic building blocks are being evaluated as laccase substrates for the generation of melanin-like polymers upon its enzymatic oxidation. The polymerization products cannot be predicted beforehand based on the selected substrate and, once the polymer is obtained, it is necessary to examine its suitability as sun care ingredient, considering not only the UV absorption capacity but also other features such as, colour, odour, stability and solubility within the sunscreen formulation. On the other hand, the cosmetic case study does not require the incorporation of the enzyme into a specific product or established reaction with predetermined parameters (pH, temperature, or unconventional substrates). Thus, the generation of a tailored laccase has not been necessary up to this point. In this context, to efficiently generate and test a panel of different melanin-like polymers within the timeframe of the OXIPRO project, a highly available, active and stable laccase is needed. In contraposition to the other oxidoreductase types used in the project, laccases have been marketed for years and different characterized options can be acquired. Therefore, this case study has been initiated with commercial laccases while exploring different substrates, mediators, and strategies for polymerization. Once these conditions are defined, and the product of interest is identified, an enzyme that works best with the appropriate substrate and mediator will be investigated.

3. Conclusions

During this Task 4.1, four different enzyme types (alcohol oxidases, carbohydrate oxidases, flavin containing monooxygenases, glucose-6-phosphate dehydrogenases) have been successfully cloned, expressed and purified in the lab, all of them in active state that allow further testing for both WP3 and WP5. The data generated in this task will allow, not only to use the enzymes in the consumer products or processes, but also to perform other tests, such as encapsulation or the microfluidic platform (WP3). Moreover, the strains generated, as well as some of the expression data, provide the basis to scale up the process and continue with Task 4.2 and 4.3, and generate enough enzyme as required in WP5.

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