# Systematic screening for the biocatalytic hydration of fatty acids from different oily substrates by *Elizabethkingia meningoseptica* oleate hydratase through a Design-of-experiments approach

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## ARTICLE INFO

## ABSTRACT

The edible plant oils production is associated with the release of different types of by-products. The latter represent cheap and available substrates to produce valuable compounds, such as flavours and fragrances, biologically active compounds and bio-based polymers. *Elizabethkingia meningoseptica* Oleate hydratases (Em_OhyA) can selectively catalyze the conversion of unsaturated fatty acids, specifically oleic acid, into hydroxy fatty acids, which find different industrial applications. In this study, Design-of-experiment (DoE) strategy was used to screen and identify conditions for reaching high yields in the reaction carried out by *Escherichia coli* whole-cell carrying the recombinant enzyme Em_OhyA using Waste Cooking Oils (WCO)-derived free fatty acids (FFA) as substrate. The identified reaction conditions for high oleic acid conversion were also tested on untreated triglycerides-containing substrates, such as pomace oil, sunflower oil, olive oil and oil mill wastewater (OMW), combining the triglyceride hydrolysis by the lipase from *Candida rugosa* and the *E. coli* whole-cell containing Em_OhyA for the production of hydroxy fatty acids. When WCO, sunflower oil and OMW were used as substrate, the one-pot bioconversion led to an increase of oleic acid conversion compared to the standard reaction. This work highlights the efficiency of the DoE approach to screen and identify conditions for an enzymatic reaction for the production of industrially-relevant products.

## 1. Introduction

The industrial production of food products for human and animal consumption generates high amount of by-products, which are differentiated depending on the specific good and are generated in different steps of the production process (Kowalska et al., 2017; Martins et al., 2017). Edible plant oils derive from seeds, germs, and fruits of certain plants, such as olive, sunflower, safflower, rapeseed, palm and soybean and their production generate large amounts of by-products (Zhou et al., 2020). Commonly, solid by-products include seed, pomace, pulp, kernel and leaf among others and they produce the so-called flour, oil cakes, meals and groats, which are considered of high importance for their low price on the market and the high content of biologically active chemicals

(Cedola et al., 2020). Moreover, a liquid residue is generated, especially from olive oil production through extraction, which is usually characterized by high salinity, low pH value, high content in nutrients and phenol derivatives and organic matter, such as triglycerides and free fatty acids (Denaro et al., 2010). In particular, olive tree (*Olea europea* L.) has been thoroughly studied for its high content in phenols derivatives, such as phenolic alcohols (hydroxytyrosol), flavonoids, secoiridoids, which have been studied for their human biological activities (*e.g.* antioxidants) (Curci et al., 2022; Micucci et al., 2015). These compounds are also present with a high content in by-products derived from production process of olive oil, such as olive mill wastewater (OMW) and pomace oil (PO) (Curci et al., 2022; Recinella et al., 2019). Other edible oil wastes are produced at the final steps of the value chain,

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after the preparation of food for the market and for preservation of edible products (Salmani et al., 2022). Waste cooking oils (WCOs) are considered renewable resources for the production of synthons, polymers and biofuels due to the presence of a mixture of triglycerides and its high availability. Due to the cooking procedure, WCOs cannot be reused for human or animal consumption. Different methods have been studied to use by-products for the production of valuable compounds.

Recently, enzymatic catalysis has been thoroughly used for the production of different products ranging from bulky products, such as polymers, to the production of valuable compounds such as active pharmaceutical ingredients due to their high regio- and stereoselectivity (Lin and Tao, 2017). Enzymes from the lyase class have shown to possess high levels of selectivity. Members of the fatty acid hydratase (FAH) family have been widely used for the transformation of unsaturated fatty acids for the production of different hydroxy fatty acids which can find applications in different fields. Specifically, Oleate hydratases (OhyA, EC. 4.3.2.53) have been used for their ability to selectively add a water molecule to the Δ9 double bond of (poly)unsaturated fatty acids, and specifically on oleic acid. This activity was reported to be useful for the conversion of natural and non-natural substrates and the creation of biocatalytic cascade reactions for the production of industrially-relevant chemicals (Demming et al., 2019, 2017; Todea et al., 2015; Zhang et al., 2020). In particular, the *Elizabethkingia meningoseptica* OhyA (Em_OhyA, PDB ID:4UIR) was the first member of the family to be characterized (Bevers et al., 2009). Nowadays, different other enzymes have been expressed and characterized due to their higher activity and lower substrate inhibition (Busch et al., 2020; Serra et al., 2021). Due to the thorough structural and biochemical characterization, the Em_OhyA is widely used as purified enzyme, as cell lysate or as *Escherichia coli* whole-cell biocatalyst. The latter is of high interest due to the use of the system as FAD reservoir, which is the cofactor needed for the biocatalytic activity (Radka et al., 2021).

Recently, we described the use of WCO-derived free fatty acids (FFA) as source of unsaturated fatty acids for the production of 10-hydroxy FFA mixture with the use of *E. coli* whole-cell biocatalyst in the presence of Tween 20 as detergent for emulsion preparation of the substrate and permeabilization of the membrane (Biundo et al., 2023a). However, different studies showed the importance of glucose as an essential additive for whole-cell systems and for fatty acids recovering. Indeed, the lack of glucose can increase the use of FFA as carbon source for cell metabolism and their degradation by β-oxidation (Demming et al., 2017). In the latter study, DoE analysis was performed on the hydration reaction on non-natural substrates, such as (*Z*)-undec-9-enoic acid for the production of (*S*)-10-hydroxyundecanoic acid, by *E. coli* whole cells carrying the recombinant enzyme Em_OhyA by using MODDE software for the analysis of the abovementioned additive, glucose, and, other additives, such as NADH and dithiothreitol (Demming et al., 2017). This allowed the production of the product on preparative scale and the stereoselective hydration of 1-decene to *S*-(+)-2-decanol by using a short fatty acid dummy substrate.

The aim of this study was the improvement of the reaction conditions for the biocatalytic conversion of WCO-derived FFA by *E. coli* whole-cell biocatalyst carrying the recombinant enzyme Em_OhyA and the identification of the critical parameters for the reaction. The Design-of-experiment (DoE) approach was carried out to analyze six different reaction conditions, namely reaction temperature, agitation, time, biocatalyst concentration, presence of detergent, substrate concentration and presence of glucose. Through a Minimum Run Factorial Design (MRFD) it was possible to identify specific conditions where oleic acid, which is the main substrate of the biocatalytic conversion, was consumed with a yield of 99 %. Biocatalytic transformation of triolein was already reported on a one-pot cascade with lipase and hydratase (Zhang et al., 2020). In this study, the identified conditions of the biocatalytic hydration of WCO-derived FFA were then applied with different triglyceride-containing substrates, namely WCO, pomace oil (PO), sunflower oil (SO), olive oil (OO) and oil mill wastewater (OMW).

## 2. Materials and methods

### 2.1. Chemicals and reagents

All chemicals and reagents used in this work were of analytical grade. Regenerated WCOs was kindly provided from Greenoil s.r.l. (Italy). Olive oil, pomace oil, sunflower oil and oil mill wastewater were kindly provided by Azienda Agricola D'Anna Francesca. Buffer components, β-D-1-thiogalactoside (IPTG), 2-Bromoacetophenone, Triethylamine, Kanamycin sulphate and Ethyl acetate (EtOAc) were purchased at the higher commercial quality and used without further purification (Sigma-Aldrich, USA).

### 2.2. Preparation of free fatty acids mixture and whole-cell biocatalyst

The free fatty acid (FFA) mixture was produced from WCOs as previously described (Biundo et al., 2023a). The biocatalyst consisted of *Escherichia coli* BL21(DE3) whole-cells containing the recombinant enzyme *Elizabethkingia meningoseptica* oleate hydratase (Em_OhyA) which was expressed as previously described (Demming et al., 2017). *E. coli* BL21(DE3) whole-cells carrying an empty pET28a+ vector were used for blank reactions to identify possible decrease of FFA by *E. coli* metabolism.

### 2.3. Biocatalytic reaction on FFA

A typical biocatalytic conversion of unsaturated FFA to hydroxy-FFA (HFFA) was carried out in 50 mL unbaffled flasks containing 10 mL of 50 mM NaCitrate buffer pH 6 supplemented with 0.4 mM Tween 20 and 10 mg mL$^{-1}$ wet cell weight (WCW) *E. coli* containing Em_OhyA and 10 mg mL$^{-1}$ WCO-derived FFA mixture at 37 °C and 200 rpm for 24 h. The reaction was carried out on thermostated orbital shakers. The reaction was stopped by the addition of 4 mL EtOAc for liquid-liquid extraction of FFA and HFFA mixture. An aliquot of 5 μL was separated into a new vial, evaporated at 80 °C and stored at −20 °C until further analysis. Standard biocatalytic conversion conditions were compared with optimal reaction conditions identified by DoE on WCO-derived FFA.

### 2.4. One-pot biocatalytic cascade reactions of oils

In order to assess the possibility to employ the identified optimal reaction conditions in a biocatalytic cascade reaction, hydrolysis and hydration reaction of different oils were carried out in one-pot with the addition of 504 U of lipase from *Candida rugosa* (Sigma-Aldrich, USA) for each reaction and subsequently the specific conditions based on the reaction to be applied. The one-pot reactions were carried out for 24 h at 200 rpm containing 0.4 mM Tween 20. For the standard reaction, the following conditions were used: Temperature: 37 °C, Biocatalyst concentration: 10 mg mL$^{-1}$, triglyceride-containing substrate concentration 10 mg mL$^{-1}$, without glucose. For the optimal reaction, the following conditions were used: Temperature: 30 °C, Biocatalyst concentration: 30 mg mL$^{-1}$, triglyceride-containing substrate concentration 5 mg mL$^{-1}$, containing 100 mM Glucose. The reactions were carried out as previously described for the WCO-derived FFA mixture, without controlling the pH of the reaction due to the presence of buffer. Both biocatalysts were added at the start of the reaction, which was stopped after 24 h by the addition of 4 mL EtOAc for liquid-liquid extraction.

### 2.5. Analysis of fatty acid content in oils and quantification

The analysis of fatty acids was realized on the starting oils following the National Renewable Energy Laboratory (NREL) method with some modifications (Wychen et al., 2013). The method consisted in a trans-esterification, followed by the extraction of fatty acid methyl esters and gas chromatography analysis. Briefly, approximately 2 mg of oil were

weighed in a HPLC-vial and mixed with 200 μl of chloroform/methanol (2:1, v/v), 300 μl of HCl 0.6 M in methanol and 25 μl of internal standard (pentanoic acid, 10 mg mL$^{-1}$). The vials were tightly sealed and put in a water bath at 85°C for 1 h. After cooling down at room temperature for 15 min, 1 mL of hexane was added, vortexed and left at room temperature for 1 h. Then, the upper phase was transferred in a new vial and 1 μl was analyzed by gas chromatography using a GC 7890 A System (Agilent Technology, USA) equipped with a flame ionization detector (FID) and a capillary column Omegawax 250 (Agilent Technologies, USA). The initial column temperature was kept at 50 °C for 2 min; the temperature was increased temperature with a ramp of 4 °C min$^{-1}$ to 220 °C and kept at the final temperature for 35 min. Total analytic time was 79.5 min and argon was used as carrier. The quantification of lipid was performed by comparing samples chromatograms with the standard. Supelco 37-Component FAME Mix (Sigma-Aldrich, USA) was used as standard. For the OMW, the extraction of lipids was obtained by adding 1 mL of hexane to 2 mL of OMW. After mixing, the mixture was let at room temperature for 1 h. Then the upper phase was transferred in a HPLC vial and the hexane evaporated by insufflating argon. Then, the same method of analysis of fatty acids was applied to the sample. The amount of oils in the OMW was gravimetrically quantified through extraction of 50 mL of OMW with 25 mL of hexane and its evaporation.

### 2.6. HPLC analysis of phenacyl esters

The mixture of FFA and HFFA present in the collected samples at the end of the reactions was analyzed by HPLC converting them into phenacyl esters through derivatization with 2-bromoacetophenone catalyzed by triethylamine, as previously described (Hagedoorn et al., 2021; Hudson et al., 1995). Briefly, dried samples were resuspended in 1 mL EtOAc and 950 μL were collected and spiked with a final concentration of 90 μM heptadecanoic acid (Sigma-Aldrich, USA) in hexane, which was used as internal standard (IS). The mixture was then evaporated at 100 °C. The dried mixture was then mixed with 25 μL of 10 mg mL$^{-1}$ 2-bromoacetophenone in acetone and with 25 μL of 10 mg mL$^{-1}$ triethylamine in acetone. The mixture was incubated in a tightly closed vial for 15 min at 100 °C. Afterwards, a volume of 3.5 μL acetic acid was added to quench the reaction by incubation of 5 min at 100 °C. The mixture was diluted in 1 mL 85 % (v/v) acetonitrile in water. The phenacyl esters were analyzed using a Waters Alliance 2695 separation module (Waters, MA, USA) equipped with a Kinetex EVO C18 column (Phenomenex, 150 mm×4.6 mm, 100 Å, 5 μm) coupled to a Waters 2996 UV detector set at 242 nm. Separation was carried out at 30 °C using 75 % (v/v) acetonitrile (solvent A) and 85 % (v/v) acetonitrile (solvent B) at a flow rate of 1 mL min$^{-1}$. Solvent A was held at 100 % for 18 min; then a linear gradient profile started reaching 100 % solvent B in 2 min and kept constant for 5 min. The initial condition was then restored in 2 min and kept constant for 10 min.

### 2.7. Design of experiment

A statistical analysis based on a factorial design was performed to determine the relative importance of different factors affecting the biocatalytic hydration conversion of unsaturated FFA, in particular oleic acid, from WCO by *E. coli* whole cell biocatalysts. Specifically, a Minimum Run Factorial Design (MRFD) was applied using Design Expert 13 software (Statease, MN, USA). The following parameters were tested: A-Temperature (30–40 °C), B-Agitation (100–200 rpm), C-Time (5–24 h), D-Biocatalyst concentration (5–30 mg mL$^{-1}$, WCW), E-Detergent, 0.4 mM Tween20 (Yes-No), F-WCO-derived FFA concentration (5–30 mg mL$^{-1}$), and G-Glucose, 100 mM (Yes-No). The model output (*Y*) is the consumption of oleic acid (%) compared to the initial content. The resulting Design of Experiment (DoE) consisted of 34 different biocatalytic reactions (runs). Center-points were included in the design to test for nonlinear relationships and to investigate the reproducibility of the experiments. The experiments were conducted in 50 mL unbaffled

shake flasks, each containing 10 mL of 50 mM NaCitrate buffer pH 6 and incubated at different temperature and agitation conditions for different time (Table 1). After the specific time of incubation, the FFA content of each run was extracted, as described above. Experimental data were fitted to a polynomial model and analyzed using an analysis of variance (ANOVA). The outputs used for the interpretation of the model were F-values together with *p*-values. F-values compare the source's mean square to the residual mean square, while *p*-values describe how probable is to detect the observed F-value if the null hypothesis is true; small *p*-values involve a rejection of the null hypothesis. F-value and *p*-value may be used together for evaluating if the overall result is significant. In our case, F and *p*-values were used to evaluate if the overall model and each variable were significant. Variance Inflation Factor (VIF), on the other hand, was used to evaluate the coefficient estimates. VIF measures how much the variance around the coefficient estimate has increased by the lack of orthogonality in the design. If the factor is orthogonal to all other factors in the model, the VIF is one.

## 3. Results and discussion

### 3.1. Identification of influencing factors

The biocatalytic hydration reaction of FFA mixture derived from WCO was performed by *E. coli* whole cell biocatalyst expressing the enzyme Em_OhyA. A DoE was applied to identify the factors that influenced the reaction based on the consumption of oleic acid, which is one of the main components of the FFA mixture from regenerated WCO, thus the production of (*R*)-10-hydroxystearic acid (10-HSA). Oleic acid was chosen due to its high solubility in ethyl acetate, which is the organic solvent used for liquid-liquid extraction. The fatty acid content of regenerated WCO was calculated by producing fatty acid methyl esters and analyzed by GC-FID. The used regenerated WCO had a high content of unsaturated fatty acids, such as oleic acid (C18:1) and linoleic acid (C18:2), of approximately 43 and 41 % w/w, respectively. Saturated fatty acids, such as palmitic acid (C16:0) and stearic acid (C18:0), had a content of 10.5 and 3.6 % w/w, respectively. A content of less than 2 % w/w was composed of other fatty acids (Fig. 1).

WCO-derived FFA were produced by hydrolysis with saponification and further acidification of the mixture. The latter procedure was previously used to perform a thorough hydrolysis of triglycerides and to remove glycerol (Biundo et al., 2023b). The presence of triglycerides, diglycerides and monoglycerides could decrease the hydration reaction efficiency due to the high specificity of members of oleate hydratase family for free carboxylic acids.

The FFA substrate was used after purification for DoE experiments. The response was reported as oleic acid consumption due to the activity of the enzyme Em_OhyA on the substrate compared to other unsaturated FFA, such as linoleic acid. In fact, it was shown that in the standard conditions when both FFAs are present, oleic acid is consumed 2.5-fold faster than linoleic acid (Fig. 2). Although linoleic acid is also used in the reaction, due to the higher conversion of oleic acid and the production of the industrially-relevant compound (*R*)-10-hydroxystearic acid, the DoE results were based on the oleic acid consumption as response factor (*Y*).

The HPLC results of the extracted FFA, after the derivatization to phenacyl esters at the end of the specific incubation time for each DoE reaction, showed that many reactions had a higher yield of conversion of oleic acid, with 15 reactions with more than 80 % conversion of oleic acid (Fig. 3, Run 1–3, 9, 14–16, 20, 21, 24–29). Interestingly, certain reaction conditions reduced the conversion of the substrate oleic acid by the enzyme (Fig. 3). In particular, run 5, 17, and 22 showed a conversion lower than 10 %.

In order to identify differences in the performance of the enzyme on oleic acid and linoleic acid consumption in reactions containing different concentrations of biocatalyst and substrate, the kinetic coefficient k$_{cat}$ was also calculated for both substrates (Fig. S1A and S1B). When both biocatalyst and substrate were used at the highest

**Table 1**
Design matrix for Minimum Run Factorial Design (MRFD).

| Runs | A Temperature (°C) | B Agitation (rpm) | C Time (h) | D Cell Concentration (mg mL$^{-1}$) | E Tween 20 (0.4 mM) | F Fatty acid Concentration (mg mL$^{-1}$) | G Glucose (100 mM) |
|---|---|---|---|---|---|---|---|
| 1 | 30 | 100 | 5 | 30 | Yes | 5 | Yes |
| 2 | 30 | 200 | 5 | 5 | No | 5 | No |
| 3 | 30 | 200 | 24 | 30 | Yes | 5 | Yes |
| 4 | 30 | 100 | 24 | 5 | Yes | 30 | Yes |
| 5 | 40 | 200 | 24 | 30 | No | 30 | Yes |
| 6 | 30 | 100 | 5 | 5 | No | 30 | No |
| 7 | 40 | 200 | 5 | 5 | Yes | 30 | No |
| 8 | 30 | 200 | 5 | 30 | No | 30 | No |
| 9 | 40 | 100 | 24 | 5 | No | 5 | No |
| 10 | 40 | 200 | 5 | 30 | Yes | 30 | Yes |
| 11 | 30 | 100 | 5 | 30 | Yes | 30 | No |
| 12 | 30 | 100 | 5 | 30 | No | 30 | Yes |
| 13 | 30 | 100 | 24 | 30 | No | 30 | No |
| 14 | 40 | 100 | 5 | 30 | No | 5 | No |
| 15 | 40 | 200 | 5 | 30 | Yes | 5 | No |
| 16 | 30 | 200 | 24 | 5 | Yes | 5 | No |
| 17 | 40 | 200 | 5 | 5 | No | 30 | Yes |
| 18 | 40 | 200 | 24 | 5 | Yes | 30 | Yes |
| 19 | 30 | 200 | 24 | 5 | No | 30 | No |
| 20 | 40 | 200 | 24 | 30 | No | 5 | No |
| 21 | 40 | 100 | 5 | 5 | No | 5 | Yes |
| 22 | 40 | 100 | 5 | 5 | Yes | 30 | Yes |
| 23 | 40 | 100 | 24 | 30 | No | 30 | No |
| 24 | 30 | 100 | 24 | 5 | Yes | 5 | No |
| 25 | 40 | 100 | 24 | 30 | Yes | 5 | No |
| 26 | 30 | 200 | 24 | 5 | No | 5 | Yes |
| 27 | 40 | 200 | 5 | 5 | Yes | 5 | Yes |
| 28 | 30 | 100 | 24 | 30 | Yes | 5 | Yes |
| 29 | 40 | 100 | 24 | 30 | No | 5 | Yes |
| 30 | 30 | 200 | 5 | 5 | Yes | 30 | Yes |
| 31 | 35 | 150 | 14.5 | 17.5 | Yes | 17.5 | No |
| 32 | 35 | 150 | 14.5 | 17.5 | No | 17.5 | No |
| 33 | 35 | 150 | 14.5 | 17.5 | Yes | 17.5 | Yes |
| 34 | 35 | 150 | 14.5 | 17.5 | No | 17.5 | Yes |
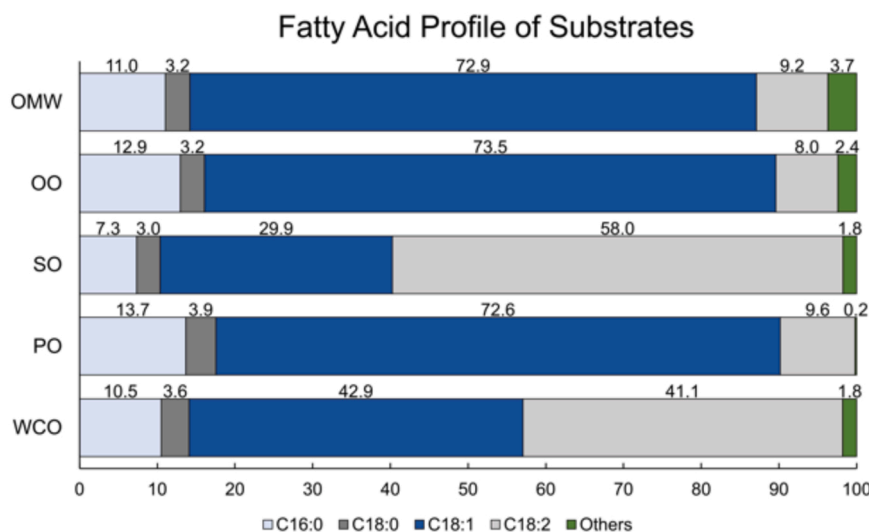


**Fig. 1.** Fatty acid composition of different substrates used in this study, namely palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2). Waste cooking oils (WCO), pomace oil (PO), sunflower oil (SO), olive oil (OO), and oil mill wastewater (OMW) were used in the study.

concentrations in the study (*D*: 30 mg mL$^{-1}$ and *F*: 30 mg mL$^{-1}$, Runs 5, 8, 10–13, 23), oleic acid conversion lower than 30 % (Fig. 3) and $k_{cat}$ values lower than 10 s$^{-1}$ for oleic and linoleic acid were measured. When both biocatalyst and substrate were used at the lowest concentrations in the study (*D*: 5 mg mL$^{-1}$ and *F*: 5 mg mL$^{-1}$, Runs 2, 9, 16, 21, 24, 26, 27) oleic acid conversions higher than 80 % were shown. Similar values were also reached in reactions containing the highest biocatalyst concentration and the lowest substrate concentration (*D*: 30 mg mL$^{-1}$

and *F*: 5 mg mL$^{-1}$, Runs 1, 3, 14, 15, 20, 25, 28, 29). In the former case, $k_{cat}$ values lower than 10 s$^{-1}$ were shown for reactions with the longest incubation time (*C*: 24 h, Runs 9, 16, 24, 26), while higher $k_{cat}$ values were shown for reactions with the shortest incubation time (*C*: 5 h, Runs 2, 21, 27). For the latter case, all reactions showed $k_{cat}$ values lower than 10 s$^{-1}$. Interestingly, in reactions containing the lowest concentration of biocatalyst and the highest concentration of substrate (*D*: 5 mg mL$^{-1}$ and *F*: 30 mg mL$^{-1}$, Runs 4, 6, 7, 17–19, 22, 30) oleic acid conversion
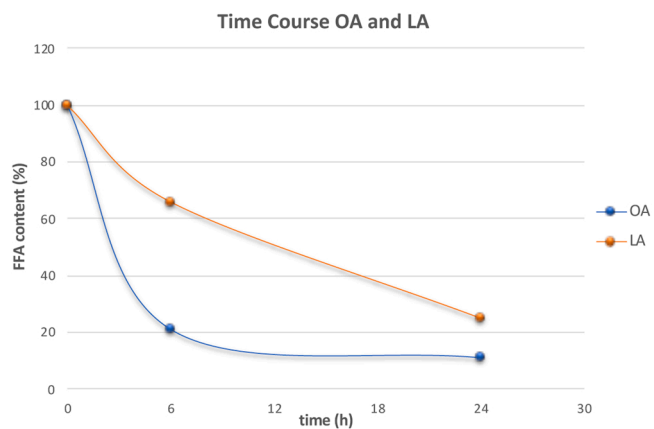
**Fig. 2.** Time course of oleic acid (OA, *blue*) and linoleic acid (LA, *orange*) consumption by the enzyme Em_OhyA starting from WCO-derived FFA.

was lower than 60 % (Run 19). However, the highest $k_{cat}$ value recorded in the experiments were measured for Runs 6 and 22, while the other runs showed $k_{cat}$ values between $10\ s^{-1}$ and $30\ s^{-1}$ for oleic acid conversion, and between $6\ s^{-1}$ and $25\ s^{-1}$ for linoleic acid conversion.

With the outputs shown in Fig. 3, the software built a model to describe the effects of experimental variables on the conversion of oleic acid. The obtained model is a non-hierarchical polynomial which takes into account combinations of the physical parameters. A non-hierarchical model was chosen to assure a better predictivity of the model considering the chemical and physical parameters taken into account (Montgomery et al., 2005).

To identify the significant effects, the interaction between factors, the "goodness of fit" and the significance and adequacy of the model, analysis of variance (ANOVA) was performed. The polynomial model responses obtained from the ANOVA of experimental data were significant with a F-value of 29.22, a *p*-value<0.0001 and a $R^2$ of 0.9435. The signal to noise was 16.33. The single factor model terms taken into account were A, C, E, F, while interactions between different factors model terms were AB, AF, BC, BE, BF, CF, DG, BDG with a *p*-value <0.01, and therefore they were all significant. The intercept (estimated of 63.28) in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor

settings. They help in identifying the relative impact of each factor. Table 2 reports variables, coefficients together with their F and *p*-values. Analysis of the variance inflation factor (VIF) showed the orthogonality of each coefficient of the selected factors to the remaining model terms. For the selected factors the VIF was close to 1 (Table 2).

The coded equation (Eq. 1) therefore is:

$$Y = 63.3 + 10.0A + 6.46\,C - 8.79E - 23.06F - 9.74AB - 6.99AF$$

$$- 6.12BC + 8.8BE - 7.15BF + 12.19CF + 13.23DG - 7.96BDG \qquad (1)$$

These results were verified by a Pareto chart (Fig. 4), which showed the *t*-values of the effects which are proportional to their degree of significance. All the effects above the *t*-value = 2.07961 are possibly significant to moderately important. With the present model, some effects reached the more rigorous Bonferroni limit = 3.6315 (Anderson, Mark and Whitcomb, 2015; Montgomery, 2020). Bonferroni limit is the threshold above which the effects that emerge are significant. The effects which most influenced the conversion of oleic acid are F, DG and CF. The other factors are significant but less impacting on the final outputs. Pareto chart showed the evaluation of the significance and type (synergistic or antagonistic) of effects.

The bar lengths are proportional to the absolute value of the estimated effects. Interpretation of the chart indicated that the strongest effect, F-concentration of WCO-derived FFA, turned out to be negative, showing an antagonistic effect on the conversion of oleic acid ($Y$),

**Table 2**
Estimates and Statistics of coefficients.

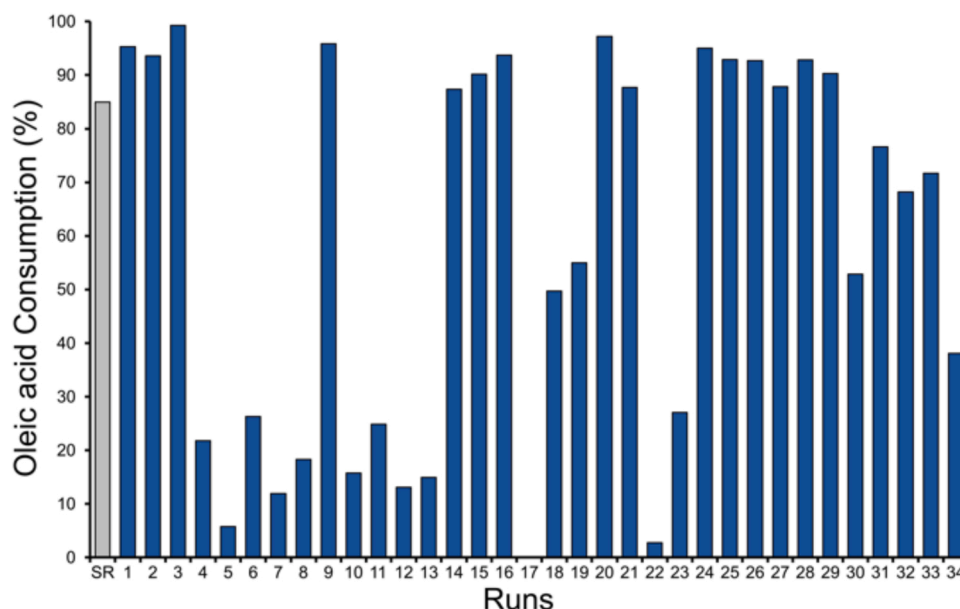| Variable | Coefficient | F-Value | *p*-value | VIF |
|---|---|---|---|---|
| **Intercept** | 63.28 | 29.22 | < 0.0001 | 1.34 |
| **A-Temperature** | 10.00 | 20.04 | 0.00 | 1.14 |
| **C-Time** | 6.46 | 9.79 | 0.01 | 1.15 |
| **E-Detergent** | -8.79 | 20.36 | 0.00 | 1.17 |
| **F-Fatty acids** | -23.06 | 121.93 | < 0.0001 | 1.32 |
| **AB** | -9.74 | 19.13 | 0.00 | 1.30 |
| **AF** | -6.99 | 10.07 | 0.00 | 1.12 |
| **BC** | 6.12 | 8.94 | 0.01 | 1.19 |
| **BE** | 8.80 | 17.36 | 0.00 | 1.17 |
| **BF** | -7.15 | 11.65 | 0.00 | 1.07 |
| **CF** | 12.19 | 35.81 | < 0.0001 | 1.19 |
| **DG** | 13.23 | 39.28 | < 0.0001 | 1.40 |
| **BDG** | -7.96 | 12.11 | 0.00 | 1.34 |



**Fig. 3.** Analysis of 34 reactions (Runs) from DoE. Relative oleic acid consumption (%) compared to standard reaction (SR, *grey*).
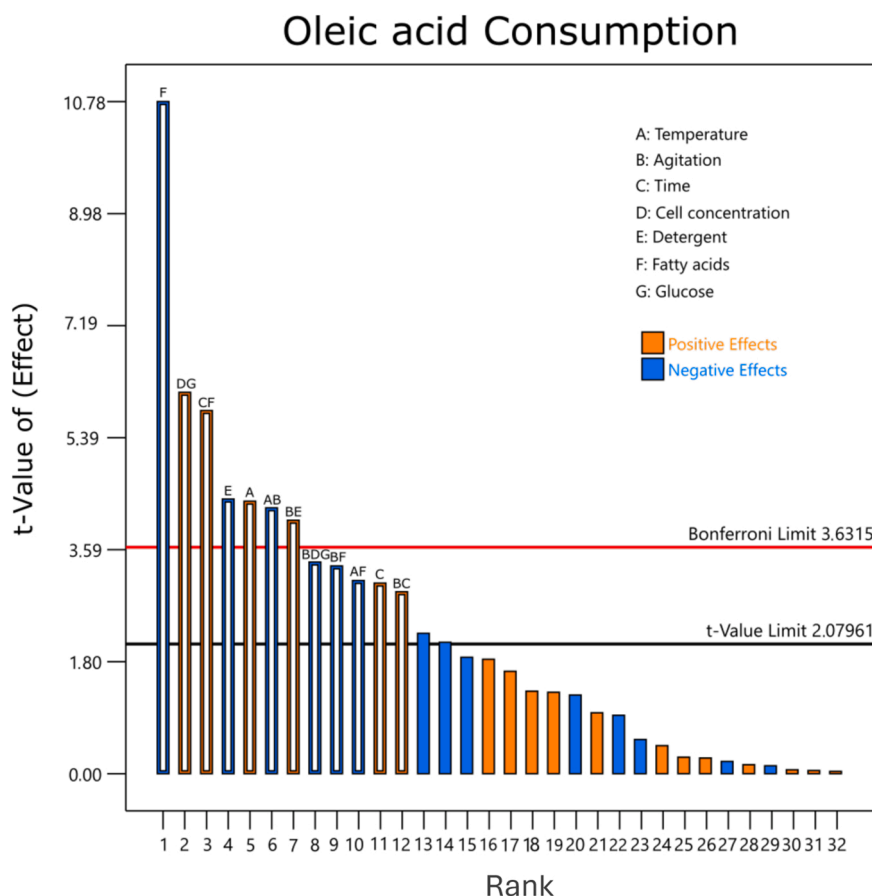
**Fig. 4.** Pareto chart of effects. Orange bars represent positive (synergic) effects and Blue bars represent negative (antagonistic) effects. The most significant values were the ones above the Bonferroni limit and were considered for the present study. Empty bars were taken into consideration while filled bars were excluded.

together with the presence of the detergent (*E*), while reaction time (*C*) and temperature (*A*) have synergic effects on *Y*. To identify the correlation between predicted and observed outputs, the points corresponding to the response (*Y*) of related runs were shown together with the perfect model response (Fig. 5). The experimental data are well fitted by the model, as shown in Fig. 5.

To test the validity of the model provided by the software Design Expert 13, the confirmation reaction was carried out in triplicates. This reaction occurred at A-Temperature: 30 °C, B-Agitation: 150 rpm, C-Time: 14.5 h, D-Biocatalyst concentration: 30 mg mL$^{-1}$, E-Yes Tween, F-FFA concentration: 17.5 mg mL$^{-1}$, G-No Glucose. The software provided a predicted response of *Y* of 48.8 % ± 10.6 and the actual value was 54.4 % ± 3.8, confirming the validity of the model.

Although different studies were performed to optimize the activity of members of the hydratase family, many are focused only on enzyme stability through immobilization and on pure oleic acid without considering the interacting effects of different reaction components or conditions (Castagna et al., 2020; Todea et al., 2015).

### 3.2. Analysis of the interactions of identified key factors

The 3D surface of Oleic acid Consumption (%) (Fig. 6) was analyzed by varying the inputs of the model. The response of the model was shown in terms of Y-Oleic acid consumption (%), shown in z axis, vs C-Time (h) and F- Concentration of WCO-derived FFA (mg mL$^{-1}$), shown respectively in x and y axis.

At the lowest levels for each experimental variable (Fig. 6A), the oleic acid consumption varied between 20 % and 60 %, when varying the range of time and fatty acids. When temperature was varied to 40 °C (Fig. 6B), a significant increase in the oleic acid consumption could be
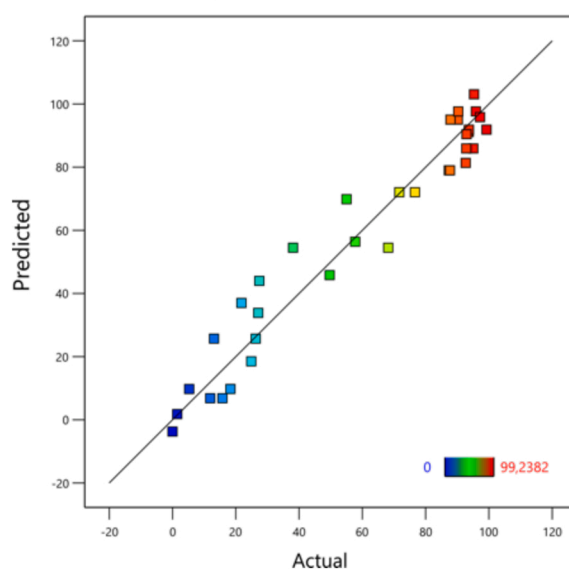


**Fig. 5.** Correlation between experimental (actual) and predicted responses. Colors of points determine the percentage of conversion of oleic acid in the experimental responses. Line represents perfect model performance.

shown from 45 %, with highest concentration of FFA of 30 mg mL$^{-1}$ and shorter tested time of 5 h, and 100 % at the lowest concentration of FFA of 5 mg mL$^{-1}$ in all the tested reaction times.
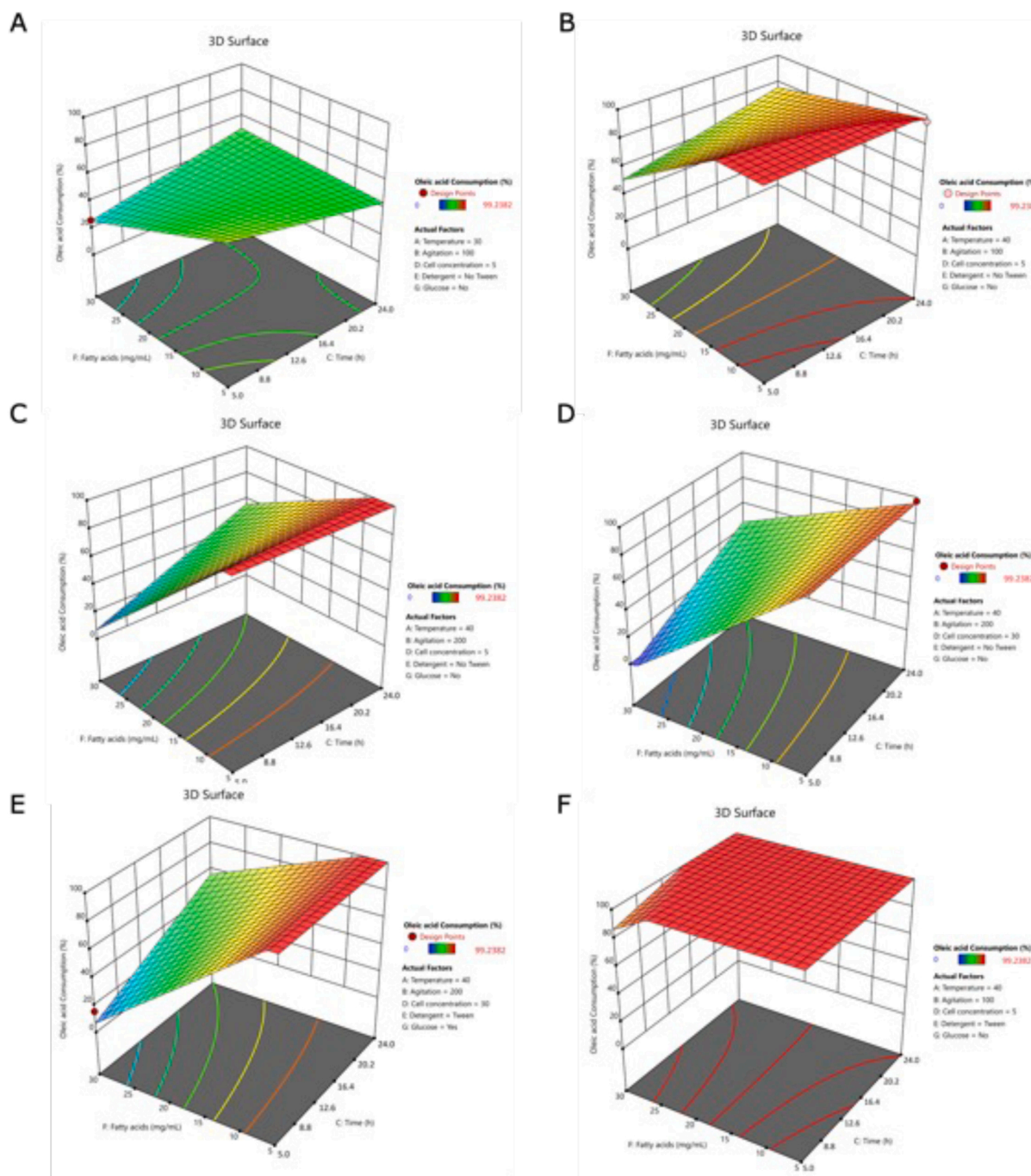
**Fig. 6.** 3D surface of conversion of oleic acid (*Y*) by varying the inputs of the model: A) A-Temperature: 30 °C, B-Agitation: 100 rpm, D-Biocatalyst concentration: 5 mg mL$^{-1}$, E-No Tween, G-No Glucose; B) A-Temperature: 40 °C, B-Agitation: 100 rpm, D-Biocatalyst concentration: 5 mg mL$^{-1}$, E-No Tween, G-No Glucose; C) A-Temperature: 40 °C, B-Agitation: 200 rpm, D-Biocatalyst concentration: 5 mg mL$^{-1}$, E-No Tween, G-No Glucose; D) A-Temperature: 40 °C, B-Agitation: 200 rpm, D-Biocatalyst concentration: 30 mg mL$^{-1}$, E-No Tween, G-No Glucose; E) A-Temperature: 40 °C, B-Agitation: 200 rpm, D-Biocatalyst concentration: 30 mg mL$^{-1}$, E-Tween, G-Glucose; F) A-Temperature: 40 °C, B-Agitation: 100 rpm, D-Biocatalyst concentration: 5 mg mL$^{-1}$, E-Tween, G-No Glucose.

With agitation set at 200 rpm, a decrease of the oleic acid consumption was shown for a large part of the experimental conditions (Fig. 6C) except for low FFA concentration. When the biocatalyst concentration was set at the maximum tested value, 30 mg mL$^{-1}$, the response further decreased, ranging from 0 and 90 % for high and low FFA concentration, respectively (Fig. 6D). Although the addition of detergent and glucose, the two categorical variables, was not shown to be incisive, an increase in oleic acid consumption was observed from 5 % to 100 % with high to low FFA concentration, respectively (Fig. 6E). Lastly, in Fig. 6F, the variables were optimized to give the best possible

results at different reaction times and FFA concentration (A-Temperature: 40 °C, B-Agitation: 100 rpm, C-Time: 5 h, D-Biocatalyst concentration: 5 mg mL$^{-1}$, E-Tween: Yes, G-Glucose: No). In this case, the software showed a high consumption for all the reaction times and FFA concentrations tested.

By summarizing the model results, the variable A-Temperature seemed to have a significant effect, and the oleic acid consumption increased together with its increase. The variable B-Agitation had also a considerable effect, especially at shorter times and lowest concentrations of WCO-derived FFA. The oleic acid consumption decreased

together with the increase of FFA concentration. Based on the model results, the optimal condition for agitation was shown to be at 100 rpm. The variable C-Time had an effect especially at high agitation. Oleic acid consumption generally increased when the reaction time was set at higher values, but it may be insignificant depending on other variables. The variable D-Biocatalyst concentration had a relevant and antagonistic effect on oleic acid consumption. The variable E-Detergent had a significant effect and reaction yield increased when Tween was added to the reaction mixture. The variable F-WCO-derived FFA concentration had a very relevant effect; it was the strongest effect in the model and had an antagonist impact on the oleic acid consumption. The variable G-Glucose had a moderate effect on the reaction yield. In general, the yields increased when it was omitted.

Results of this model are consistent with what was previously observed. In fact, Seo et al. showed that the yield of the conversion reaction of ricinoleic acid to 10,12-Dihydroxystearic acid by the oleate hydratase from *Lysinibacillus fusiformis* was high at the lowest tested concentrations of substrate. On the other hand, they observed that the yield of product is higher when increasing the concentration of biocatalyst to a maximum. In our work, instead, the consumption of oleic acid is increased when biocatalyst is added at low concentrations to the reaction mixture (Seo et al., 2013). Previously reported work studied the biotransformation of oleic acid to 10-hydroxystearic acid by a hydratase from *Lactococcus garvieae* and observed that the enzyme has an optimum of relative activity at 30°C, similarly that in this work. Furthermore, the relative activity has an optimum when substrate concentration is at 30 g L$^{-1}$, while we observed a better response in terms of substrate consumption at 5 g L$^{-1}$. The reported experiments were performed with pure oleic acid, while, in our experiments, considering that different FFA are employed, possible inhibitory effects on the enzyme, especially at higher FFA concentration, may be involved. In fact, WCO-derived FFA have a similar concentration of oleic acid and linoleic acid, and a smaller concentration of saturated fatty acids (Fig. 1).

### 3.3. Cascade biocatalytic reaction on different substrates

Considering the overall response of the model, a reaction which resulted in an optimal oleic acid consumption was chosen from the list of reactions performed for the DoE. The chosen design was the Run 3, which are herein described as optimal reaction conditions (OR), consisting of A-Temperature: 30 °C, B-Agitation: 100 rpm, C-Time: 24 h, D-Biocatalyst concentration: 30 mg mL$^{-1}$, E-Tween: Yes, G-Glucose: Yes.

The oleic acid consumption resulting from this reaction was compared to the standard reaction (SR) conditions carried out and applied to several substrates (Biundo et al., 2023).

In order to test the optimal conditions on untreated substrates, WCO, PO, SO, OO and OMW were used as collected in one-pot biocatalytic reactions containing the lipase from *Candida rugosa* (CrL), which catalyzes the hydrolysis of triglycerides into FFA, and *E. coli* whole-cell biocatalyst containing Em_OhyA for the hydration of unsaturated FFA (Fig. 7).

The activity of CrL on triglycerides is well-known and previous

reports showed biocatalytic cascade reactions with the mentioned biocatalysts (Zhang et al., 2020). The substrates were first characterized in terms of fatty acid content as FAME by GC-FID (Fig. 1). The selected oil-based substrates showed a similar content in saturated fatty acids, ranging from 10.3 and 17.6 %. On the other hand, the unsaturated fatty acid, in particular oleic acid and linoleic acid, content was different for each substrate. In fact, the content of oleic acid (C18:1) was higher for PO and OO, with a value of 72.6 and 73.5 %, respectively. On the other hand, a lower oleic acid content was present in SO, with a value 30 %. OMW showed a high content of oleic acid similar to PO and OO of approximately 73 %. Contrarily, linoleic acid (C18:2) showed the opposite trend, with the highest percentage in SO. OMW is a diluted effluent from the olive oil industry that contains a high water content, thus a very diluted solution of triglycerides of approximately 0.15 %. This substrate was chosen to identify the possibility to use industrial by-products with environmental and economic issues. The content of unsaturated fatty acids present in each reaction is reported in Table 3.

These substrates were then used as collected for the one-pot biocatalytic cascade with the use of CrL and Em_OhyA. The amount of oil-based substrates was accordingly used to the conditions stated in the standard reaction (SR) or in the run 3 of DoE (optimal reaction, OR) (Table 4).

One-pot biocatalytic reactions with the different substrates showed an interesting trend (Fig. 8). In fact, the oleic acid content in untreated oil-based substrates, specifically WCO, PO, SO, and OO, with the standard reaction (SR) conditions was consumed from 35, for OO, to almost 60 %, for WCO. When optimal reaction (OR) conditions were used, oleic acid in WCO and SO was further consumed to 98 % and 64 %, respectively.

Interestingly, this improved conversion was not shown for PO and OO, in which, when OR conditions were applied, oleic acid consumption was similar and lower compared to SR conditions, respectively.

Two possible reasons for this inhibition of the biocatalytic activity could be given by the fatty acid content of the oil-based substrates. On the one hand, it was previously reported that members of the oleate hydratase family could be inhibited by higher oleic acid content (Castagna et al., 2020). The different fatty acid ratio can produce an inhibitory effect on the conversion of the main substrate, oleic acid. In fact, a similar increasing trend was shown for oil-based substrates that contained a similar content of oleic acid and linoleic acid, namely WCO and

**Table 3**

Unsaturated fatty acid content in one-pot biocatalytic conversion using standard reaction conditions (SR) and optimal reaction conditions (OR).

| | SR | | OR | |
|---|---|---|---|---|
| | OA (mg L$^{-1}$) | LA (mg L$^{-1}$) | OA (mg L$^{-1}$) | LA (mg L$^{-1}$) |
| WCO | 4290 | 4110 | 2145 | 2055 |
| PO | 7260 | 960 | 3630 | 480 |
| SO | 2990 | 5800 | 1495 | 2900 |
| OO | 7350 | 800 | 3675 | 400 |
| OMW | 110 | 14 | 55 | 7 |
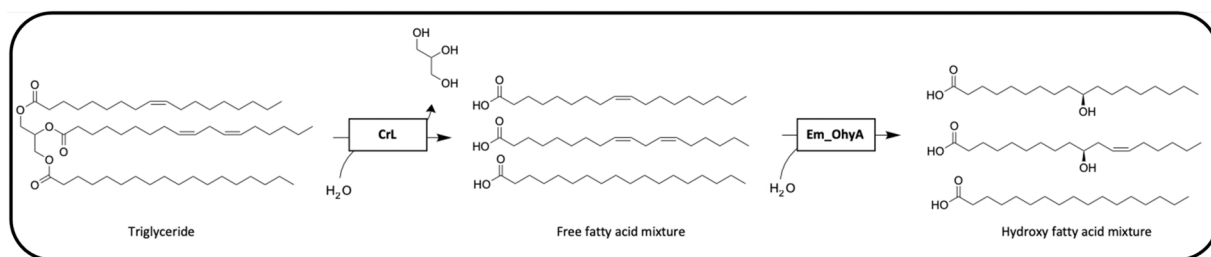


**Fig. 7.** One-pot Biocatalytic cascade reaction for the transformation of oil-based substrates into hydroxy fatty acid mixture with the hydrolysis of triglycerides by Lipase from *Candida rugosa* and the release of glycerol and the hydration of unsaturated free fatty acids from the free fatty acid mixture by Em_OhyA to produce hydroxy fatty acid mixture.

**Table 4**
Summary of reaction conditions for standard reaction (SR) and for optimal reaction (OR).

| Reactions | Temperature (°C) | Agitation (rpm) | Time (h) | Cell Concentration (mg mL$^{-1}$) | Tween 20 (0.4 mM) | Fatty acids Concentration (mg mL$^{-1}$) | Glucose (100 mM) |
|---|---|---|---|---|---|---|---|
| SR | 37 | 200 | 24 | 10 | Yes | 10 | No |
| OR | 30 | 200 | 24 | 30 | Yes | 5 | Yes |
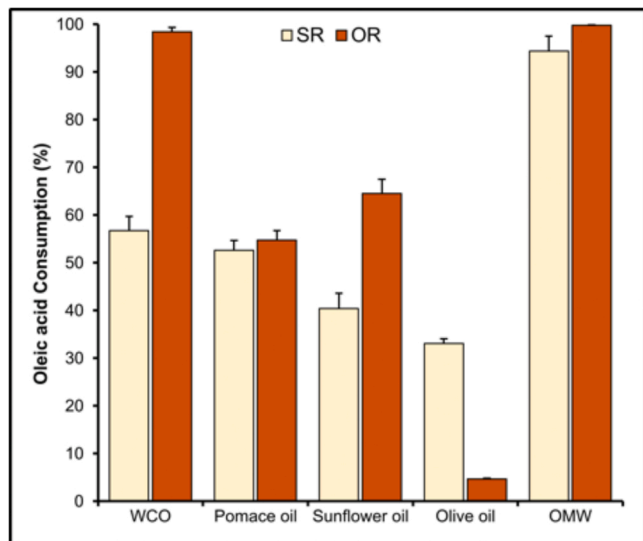


**Fig. 8.** Comparison of Oleic acid consumption (%) with cascade catalytic reaction of standard reaction (SR) and optimal reaction (OR) in different substrates.

SO. These two substrates showed a ratio of oleic acid:linoleic acid of 1 and 0.5, respectively. On the other hand, PO and OO had a ratio of oleic acid:linoleic acid of 7.5 and 9.2, showing a higher content of oleic acid over linoleic acid.

On the other hand, the lower temperature (30 °C) in OR conditions could reduce the melting of saturated fatty acids, such as palmitic acid and stearic acid, which could inhibit the reaction by creating agglomeration with unsaturated fatty acids. Moreover, PO and OO had a saturated fatty acid content of 17.6 and 16.1 %, respectively. Contrarily, WCO and SO showed only 14.1 and 10.3 % of saturated fatty acid content, respectively.

Overall, these results show that several oleic acid-rich sources may be successfully employed to perform a one-pot cascade catalytic reaction involving CrL and Em_OhyA to obtain HFA. Furthermore, the DoE approach was able to screen and identify optimal conditions that were efficient in increasing the reaction yield, especially in oleic-acid sources such as WCO, SO and OMW.

## 4. Conclusions

In this work, we screened the conditions involved in the biotransformation of fatty acids from WCO to 10-hydroxy fatty acids through a whole-cell biocatalyst. The screening occurred through a DoE approach which resulted in a model able to show the impact of the experimental variables temperature, agitation, time, biocatalyst concentration, presence of detergent, WCO-derived FFA concentration and presence of glucose. The obtained model was a significant polynomial and non-hierarchical model (F-value of 29.22, $p$-value$<0.0001$, $R^2$ 0.9435). Among the 34 tested reactions, Run 3, consisting of A-Temperature: 30 °C, B-Agitation: 100 rpm, C-Time: 24 h, D-Biocatalyst concentration: 30 mg mL$^{-1}$, E-Tween: Yes, F-Fatty acid concentration: 5 mg mL$^{-1}$, G-Glucose: Yes, showed the highest conversion of oleic acid, one of the FFA present in the mixture.

In a second part of the work, a one-pot biocatalytic cascade reaction was set up involving CrL and Em_OhyA. The biocatalytic cascade reaction was carried out with the experimental conditions identified with the DoE approach and was tested on several oleic acid-rich substrates, identifying WCO, SO and OMW as best sources to perform the reaction. Future perspectives should consider the use of the substrates of different pH values, such as pH 8, in order to test the higher solubility of FFA at these conditions. The latter could increase the accessibility of the substrate to the active site of the enzyme. Moreover, based on the high variability of the feedstock, due to geographical regions and other factors, further studies should focus on the blending of triglyceride-rich substrates to identify possible ideal mixtures which could have a FFA profile, and in particular the oleic acid/linoleic acid ratio, for the condition transferability to allow high conversions of unsaturated FFA and on the creation of fixed bed bioreactors where both enzymes could act in parallel.

## CRediT authorship contribution statement

**Francesca Scargiali:** Funding acquisition, Data curation. **Gennaro Agrimi:** Writing – review & editing, Writing – original draft, Validation, Resources. **Annabel Serpico:** Formal analysis. **Cosetta Ciliberti:** Validation, Formal analysis, Data curation. **Marianna Ciaccia:** Validation, Formal analysis, Data curation. **Serena Lima:** Writing – review & editing, Writing – original draft, Validation, Methodology. **Antonino Biundo:** Supervision, Project administration, Funding acquisition, Conceptualization. **Isabella Pisano:** Writing – review & editing, Writing – original draft, Supervision.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

Data will be made available on request.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiotec.2024.06.016.

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