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Enzymatic synthesis of Hydroxytyrosol from Oleuropein for valorization of an agricultural waste

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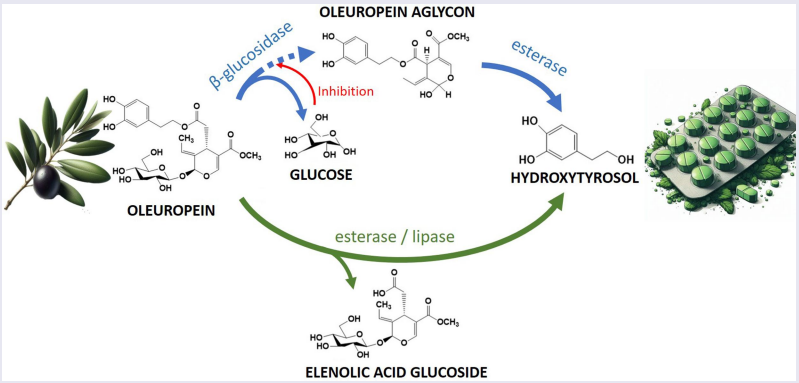
ABSTRACT

Oleuropein (OP) is an appreciated compound present not only in fruits but also in leaves of olive trees, which can be transformed into hydroxytyrosol (HT), a substance with high antioxidant activity. In this work, the transformation of an agricultural residue containing OP (olive leaves or wastewater from mills) to the high added value compound HT is accomplished through different enzymatic strategies. Different enzymes were used, immobilized on various supports by diverse binding forces: beta-glucosidase encapsulated in siliceous material, esterases and lipases immobilized on hydrophobic supports (octyl-functionalized amorphous silica and periodic mesoporous organosilica), and esterase immobilized on amine-functionalized ordered mesoporous silica. All these biocatalysts were tested for oleuropein hydrolysis through two different reaction approaches: a) split of glucosidic bond catalyzed by beta-glucosidase (β-glu), followed by hydrolysis of the aglycon and further ester hydrolysis. 5 mg·mL^{-1} of β-glu fully hydrolyzed 5 mM OP at pH 7 and 50°C in 7 days, and further enzymatic hydrolysis of the aglycon yielded near to 0.5 mM HT in the best conditions tested. b) via direct hydrolysis of the ester bond to produce hydroxytyrosol in a one-step reaction using esterases or lipases. The latter reaction pathway catalyzed by lipase from *Penicillium camemberti* immobilized on octyl-silica (4 mg·mL^{-1}) at 35°C and pH 6 directly produced 6.8 mM HT (1 mg·mL^{-1}), transforming in 12 days near to 30% of the initial 25 mM OP from a commercial olive leaves extract.



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Oleuropein; hydroxytyrosol; immobilized enzymes; waste valorization; enzymatic transformation

Introduction

Polyphenols are polar organic compounds that can be readily found in wine and olive oil [1,], contributing to the benefits of the Mediterranean diet [2,3]. Hydroxytyrosol is a polyphenol present in olive fruits and leaves with great antioxidant activity, much higher than that of oleuropein and vitamins C and E [4]. It offers a wide range of beneficial effects on health such as anti-inflammatory power, reduction of the risk of coronary heart disease [5] or reduced risk of atherosclerosis. As free radical scavenger [6] hydroxytyrosol is easily absorbed by the intestine and transferred to

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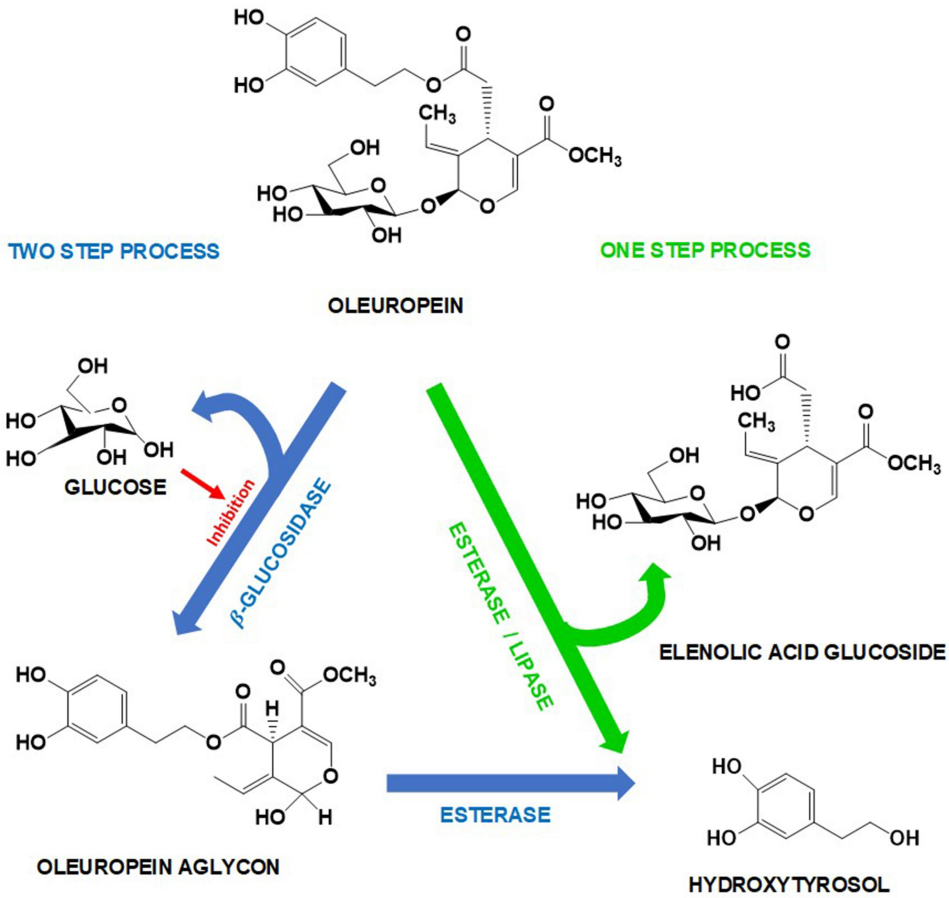
blood to palliate and prevent toxic effects produced by the accumulation of free radicals in the tissues, and it inhibits the oxidation of human LDL [7]. Recently, the possible effects of HT on the mitochondrial disfunction observed in Alzheimer's disease are being studied [8]. With such a variety of beneficial health effects, it is easy to imagine the relevance of the market of this kind of antioxidants, especially in the field of food additives, dietary supplements or anti-aging products [9].

Olive fruits and leaves contain the secoiridoid Oleuropein (OP) and very low amounts of its derivative Hydroxytyrosol (HT) (Scheme 1) [10]. Physico-chemical processes for olive oil refining result in HT degradation, therefore only the virgin olive oil (this is, obtained by just mechanical extraction), is rich in HT from olive fruits. In order to obtain HT by industrial methods from mixtures of polyphenol extracts from olive products or residues, techniques like elution after adsorption, water or supercritical CO_2 extraction or nanofiltration followed by reverse osmosis [11] are used yielding HT in ranges between 1.2 and 18%. Through supercritical extraction and nanofiltration [9], concentration of HT in the extracts increases, and the value per kg of HT in these extracts raises exponentially.

The availability of OP in olive leaves is an opportunity to transform the agricultural waste generated by annual pruning and destined for burning into a high added value product. Besides, OP is also present in water from olive milling as a contaminant. We propose to obtain hydroxytyrosol from oleuropein-containing agricultural wastes from olive cultures, mainly leave extracts obtained from tree pruning. The sustainability of this process can be additionally improved by introducing the biocatalytic alternative, especially considering the high costs, complexity, yield and energy consumption of some of the aforementioned industrial methods [12].

The first description of the use of beta-glucosidase (β-glu) to obtain HT from OP via cleavage of the glucosidic bond was performed in 1994 by Ciafardini [13]. Based on this work,



Scheme 1. Enzyme catalyzed reaction pathways tested in this work for the production of hydroxytyrosol from oleuropein.

Briante [14] and [15] reported studies using immobilized β-glu to catalyze OP hydrolysis resulting in oleuropein aglycon and glucose. This aglycon can be later hydrolyzed through different strategies: heat treatment [16–18] or enzymatic hydrolysis with an esterase. Since then, many studies have been performed based in this strategy. β-glu alone [18] or acting synergistically with esterases to hydrolyze the ester bond between HT and elenolic acid glucoside or OP aglycon have been explored. Nikolaivits [19] used β-glu and lipase to fully degrade 1.5 mM OP. Although cellulases have also been used either free [20] or immobilized and used in reactor column [21], the aglycon is mostly obtained with β-glu. The glucose released in this reaction is an inhibitor of this enzyme, which considerably limits the starting concentration of OP. However, not many data regarding this inhibition can be found, an exception being Ciafardini [13] who reported a 40–50% inhibition of β-glu by 2% glucose.

Microorganisms with both glucosidase and esterase activities have also been described to perform this transformation: Lactobacillus plantarum strains [13,22] or different lactic acid bacteria [23], Aspergillus niger [24], Wickerhamomyces anomalus and Lactiplantibacillus plantarum [25]. W. anomalus BS81 belongs to the collection of the Department of Agriculture, Food and Environment of the University of Catania [26], where Palmeri compared the effect of the whole W. anomalus with commercial β-glu and esterase [27].

To the best of our knowledge, transformation of OP into HT has not been approached using just one enzyme with esterasic activity to split the oleuropein molecule into hydroxytyrosol and glucosilated elenolic acid in a one-step reaction. This reaction is addressed in this work testing esterases and lipases.

Lipases commercially available were studied and compared to an esterase obtained from an efficient esterase producing strain. This organism was isolated from naturally fermented olive brine, which means that the enzymatic HT production can be performed with a catalyst available from the same process that produces the residue.

The use of immobilized enzymes does not only favor the reutilization of the biocatalysts, but also and most interestingly, it allows to design and choose the best support material to improve the characteristics of the enzyme-support system. The immobilization via non-covalent enzyme-support link has proven successful to obtain catalysts with high enzyme loading and activity values. Leaching can be controlled by a smart design of the support that fulfills two main conditions: a) Textural properties, with appropriate shape and size of pores where uniform pores provide connectivity of the porous network and facilitate the diffusion of the enzyme, and pore size matching enzyme dimensions providing confinement of the enzyme inside them; and b) functionalization of the surface of the support with chemical groups to allow a high chemical affinity with those on enzyme surface. This strong attraction together to the close vicinity of groups from support contributes to retain the enzyme within the pores and to preserve it from being released. Therefore, the catalysts can combine the advantages of those prepared by both, covalent and non-covalent bonding [28]. On the other hand, one-pot enzyme immobilization by synthesizing the siliceous support in the presence of the enzyme is possible after adjusting synthesis conditions to obtain these materials while preserving enzyme activity. In-situ β-glu/silica used in this work displays a cage/window shape of the pores, where large cages able to accommodate one enzyme molecule are connected by narrow windows, smaller than enzyme dimensions [29]. The enzyme in these materials cannot be released at all.

In this work, enzymes with different characteristics are used: β-glucosidase with a large molecular size, an esterase with low isoelectric point, as well as another esterase and lipases with hydrophobic domains on their external surfaces. This variety enables to apply different immobilization strategies. Thus, supports with tailored textural and surface chemical properties were synthesized and employed in this work to immobilize the different enzymes used, according to the properties of each of them. Octyl-functionalized amorphous silica (OAS) [30], and periodic mesoporous organosilica (PMO) with ethylene bridging groups [31] were the materials used for lipase immobilization via hydrophobic interactions with the surface of the enzyme. Esterase E was also immobilized on OAS. Beta-glucosidase was immobilized by in situ

encapsulation in a siliceous mesoporous material [29]. An esterase with low isoelectric point [32] was immobilized on positively charged siliceous mesoporous SBA-15-NH_2 (aminopropyl-fuctionalized ordered mesoporous silica with SBA-15 structure) via electrostatic interactions.

In this work, a battery of tailor-made biocatalysts is tested for the study under mild reaction conditions of the specific enzymatic transformation of OP from agricultural wastes into HT, with high added value.

Materials and methods

Materials

Amorphous Silica MS-3030 was a kind gift from PQ Corporation (USA), n-octyltriethoxysilane 95% (OTES) and ammonium fluoride 98% were obtained from Alfa-Aesar, 4-nitrophenylacetate (p-NPA), 4-nitrophenyl β-D-glucopyranoside (p-NPG), 1,2-bis(trimethoxysilyl)ethane (BTMSE), Pluronic P123, Pluronic F-127, tetraethoxysilane 98% (TEOS), 1,3,5-trimethylbenzene 98% (TMB), 1,3,5-triisopropylbenzene 95% (TIPB), oleuropein 80% and hydroxytyrosol 98% were purchased from Sigma-Aldrich. Olive leaves extract containing 20% Oleuropein was kindly donated by Monteloeder (Elche, Spain). Potassium chloride, ethanol absolute and hydrochloric acid 37% were obtained from Panreac. HPLC grade acetonitrile, obtained from Honeywell, and phosphoric acid, obtained from Sigma-Aldrich, were used for HPLC analyses.

Enzymes

Beta- Glucosidase

Novozymes 188 from *Aspergillus niger* was kindly donated by Novozymes Spain. The extract was first filtrated with a nylon membrane (Millipore 0.45) and then ultrafiltration was performed using a 100 KDa membrane.

Esterases:

LAE6 is a promiscuous esterase isolated by M. Ferrer and col [32].

Esterase (E) was extracted from *Wickerhamomyces anomalus* BS81 through beads milling (3 µm). Briefly, 1 g of beads was added to 1 g of lyophilized cells mixed with 4 mL of Citrate-Posphate buffer alternating 20 cycles for 5 min in an ice bath. The supernatant was purified in the retentate by ultrafiltration with a 300 kDa membrane (Millipore). This enzyme was produced and isolated by Restucia et al. [26].

Lipases

TL (from *Thermomyces lanuginosa*) and CaLB (from *Candida antarctica*) were kindly donated by Novozymes Spain. Lipases C (AY Amano 30SD from *Candida rugosa*), G (Amano 50 from *Penicillium camembertii*), and D (DF Amano 15 from *Rhyzopus oryzae*) were kindly donated by Amano Enzymes (Japan).

Protein quantification was performed according to Bradford assay [33].

Immobilization of the enzymes

In situ encapsulation of beta-glucosidase

The biocatalyst was prepared by the *in situ* encapsulation method developed in our group [29,34]. The enzyme solution used was Novozyme 188, a β-glu extract ultrafiltered as described above. TEOS (tetraethoxysilane) was used as the silica precursor, Pluronic F-127 as the surfactant template and TMB (trimethylbenzene) as swelling agent. The procedure to synthetize the siliceous material in the presence of the enzyme is described in the Supplementary Information section (SIS).

Post synthesis immobilization of esterases and lipases

Obtaining the functionalized support materials is described in SIS. Briefly, an amorphous silica with average pore diameter around 30 nm was grafted with octyl groups (hereinafter octyl amorphous silica is designed OAS). SBA-15 was obtained as described in SIS and grafted similarly with amine groups. This material displays parallel cylindric channels with pore diameters around 10 nm [28]. And finally, Periodic Mesoporous Organosilica (PMO) bearing ethylene bridging groups was also synthesized [31].

For enzyme immobilization, the respective functionalized supports were suspended in enzyme solutions at the appropriate pH and gently stirred. Aliquots of the supernatants were withdrawn and assayed at different times. When the activity of

supernatants was zero or diminished to a constant value, the immobilization was terminated. The suspensions were then filtered, washed with the same buffer and dried. The final protein content of the supernatants was tested by Bradford analysis, which enables to calculate the enzyme loading (EL) in terms of mg of enzyme per gram of solid biocatalyst. The respective dried biocatalysts were suspended (10 mg·mL^{-1}) and assayed for catalytic activity in order to calculate the activity in units per gram of catalyst (AG^{-1}). The ratio AG^{-1}/EL is defined as the Catalytic Efficiency, which is the activity units per mg of immobilized enzyme.

- *Lipases and Esterase E*: The immobilization of these enzymes was performed as described in previous works of the group [35]. Enzyme solutions were prepared to achieve a maximum initial loading of 100 mg of enzyme per gram of support. Either Octyl Amorphous Silica (OAS) or PMO were suspended in enzyme solutions at pH 6 in 50 mM potassium phosphate. The driving forces of immobilization are the hydrophobic interactions of the enzyme surfaces with the octyl or ethylene groups of these support materials, respectively.
- *Esterase LAE6*: This enzyme was immobilized on an ordered mesoporous siliceous support with its surface coated with aminopropyl pendant groups (SBA-15-NH_2) as described elsewhere [32]. The esterase was immobilized through electrostatic interactions at pH 7.0 in 0.1 M HEPES buffer. Enzyme solution was prepared to achieve a maximum load of 50 mg of enzyme per gram of support, and the final enzyme loading achieved was 48 mg esterase per gram of silica. The catalyst will be named LAE6/SBA15NH_2.

Catalytic activity assays

Routine assays of the activities of the enzymes were carried out spectrophotometrically on an Agilent 8453 UV–visible diode array spectrophotometer (Agilent Technologies) equipped with stirring device and constant temperature capability.

Beta-Glucosidase

The enzyme activity was tested using p-NPG. To a cuvette containing 1.5 mL of 50 mM phosphate buffer pH 5, 0.5 mL of 10 mM p-NPG prepared in the same buffer were added. Aliquots of enzyme solution or suspension were added at 35°C under stirring and the increase of absorbance at 405 nm during 5 min was measured. The molar extinction coefficient ε of p-nitrophenol at pH 5.0 and 35°C is 240 M^{-1}·cm^{-1}.

Esterases and lipases

Assays were performed by following the increment of absorbance per minute at 348 nm registered by the addition of 50 µL of enzyme solution or suspension on 1.9 mL 0.4 mM p-NPA in 50 mM phosphate buffer pH 7.0 at 25°C. The molar extinction coefficient ε of p-nitrophenol at pH 7.0 and 25°C is 5150 M^{-1}·cm^{-1}.

Leaching tests

Immobilized esterases and lipases were suspended in 50 mM phosphate buffer pH 6.0 at 35°C. The solid/liquid ratio of these suspensions was 1.25 mg·mL^{-1} as previously described [35]. β-glu was suspended in 50 mM phosphate buffer at pH 5.0 maintaining the same ratio.

At different times aliquots of suspensions were withdrawn and centrifuged and the supernatants were tested to determine the concentrations of released proteins by Bradford assay [33].

Cleavage of glucosidic bond of oleuropein by beta-glucosidase

The following variables of reaction were studied: oleuropein in concentrations between 2.5 and 20 mM in a total volume of 10 mL, pH between 5.0 and 7.0, temperature 40 or 50°C, and with 50 or 100 mg of immobilized β-glu (that is, 5 or 10 mg biocatalyst per mL of reaction mixture suspension). Samples were incubated in individual sealed vials to prevent evaporation. At certain times

aliquots (one vial) were taken, cooled and assayed in HPLC in the conditions described below.

Hydrolysis of ester bond of oleuropein aglycon

- Non-enzymatic: The supernatant of the reaction of oleuropein hydrolysis was heated at 60 or 70°C under stirring and aliquots were withdrawn and analyzed by HPLC.
- Enzymatic: 20 mg of immobilized esterase (LAE6/SBA15NH$_2$) were added to 5 mL supernatant from the reaction of 5 mM oleuropein hydrolysis. The suspension was incubated at 25°C under stirring. Aliquots of the suspension were withdrawn and centrifuged to analyze the supernatants by HPLC. Alternatively, the same conditions were applied to 5 mM pure OP to test the first approach to direct HT obtention.

One-step oleuropein hydrolysis with esterase E and lipases

Reactions were carried out in a Precision Scientific 360 Orbital Shaker Bath. Conditions studied were as follows: Temperature of the bath: 30–40°C; initial OP concentration: 12–25 mM (from leaves extract); enzyme concentration: 0.09–0.2 mg·mL^{-1} suspension; catalyst concentration: 1.4–4 mg·mL^{-1} suspension, and pH: 6.0–8.0. The reaction suspensions prepared were split in separate 1 mL vials to avoid evaporation with each sampling. One vial of each set of conditions was taken every 24 h and the respective supernatants were analyzed by HPLC for HT and OP.

Set of HPLC conditions

Pure commercial oleuropein and hydroxytyrosol were used to determine their retention times in the different HPLC tests and to perform their respective calibration curves. Several mixtures of solvents were tested in order to optimize the assays.

Chromatographic system HPLC-UV-visible Agilent Technologies 1260 Infinity was used, with an Agilent Eclipse Plus reversed phase column C18 (15 cm × 4.6 mm, 5 µm). Separation was achieved by a gradient elution. The solvent systems used for separation were (A) phosphoric acid 0.02 M, and (B): acetonitrile, and the flow rate was 1 mL·min^{-1}. The column temperature was set to 25°C.

The elution conditions used to follow the reaction of cleavage of glucosidic bond of oleuropein were: initial A – B (90:10); for 3.5 min A – B (90:10); in 0.5 min A – B (80:20); for 16 min A – B (80:20); in 2 min A – B (50:50); in 3 min, A – B (0:100); for 5 min A – B (0:100); in 1 min A – B (50:50); for 2 min A – B (50:50); finally in 1 min A – B (90:10). Absorbance at 280 and 240 nm were recorded; hydroxytyrosol and oleuropein were identified at 280 and 240 nm using oleuropein (80% oleuropein Sigma-Aldrich) and hydroxytyrosol (98% Hydroxytyrosol Sigma Aldrich) as references. The total injection volume was 20 µL. The concentrations of the compounds for calibration curve were 5 mM for both of them. Retention times were 13.9 min for oleuropein and 2.9 min for hydroxytyrosol (see Figure S1). Elution conditions were slightly modified to follow the direct esterification reaction of oleuropein. Initial A-B (90:10), 3.5 min after (80:20), 16.5 min after (50:50), 2 min after (0:100), 8 min after (50:50), after 4 min (90:10) for 1 last minute. Retention times in these conditions were (HT: 3.1 min, and OP: 14.8 min).

Results and Discussion

Characterization of biocatalysts

Immobilized catalysts

The enzyme loadings and catalytic activities of the different biocatalysts obtained are summarized in Table 1. The initial amounts of soluble enzymes offered to 1 g of the supports were 100 mg, except for esterase LAE6, which was 50 mg, so the maximal theoretical enzyme loadings were 100 mg·g^{-1} (50 mg·g^{-1} for esterase LAE6). Catalytic activities displayed are calculated according to the assays described in section 2.4 and expressed in enzyme units per gram of final biocatalyst (U·g^{-1}). Specific activity is also expressed as Catalytic efficiency, in units per milligram of immobilized enzyme (U·mg^{-1}).

Leaching tests

Non-covalent immobilization of enzymes is usually easier and faster than covalent bonding, and allows better preservation of catalytic activity. However, the

Table 1. Enzyme immobilization results. Activity of lipases and esterases in p-npa units; β-glu in pNPG units.

Enzyme	Support	Duration (h)	Enzyme loading ($mg \cdot g^{-1}$)	Biocatalyst activity ($U \cdot g^{-1}$)	Cat. Effic. ($U \cdot mg^{-1}$)
Lipase TL	OAS	24	3	211	4.45
Lipase CaLB	OAS	3.5	98	358	3.64
Lipase C	OAS	3.5	48	198	3.69
Lipase G	OAS	4	70	10	0.10
Lipase D	OAS	2.5	49	226	4.64
Lipase CaLB	PMO	4	67	100	1.08
Lipase G	PMO	4	51	44	0.45
β-glu	IS	4	100	0.1	1.54
Esterase E	OAS	24	100	2	0.02
Esterase LAE6	$SBA15NH_2$	24	50		

nonpermanent nature of non-covalent forces allows desorption of the enzyme after immobilization, in contrast to the irreversible nature of covalent bonding. That is why the leaching of enzymes in catalysts with non-covalent binding of the enzyme must be tested, the results are shown in Figure 1.

Beta-Glucosidase: As described in the supplementary information section, the enzyme is encapsulated in spherical cavities 8.5 nm in diameter of this porous silica support interconnected through openings less than 4 nm wide, too narrow to permit outwards diffusion of the enzyme. This means that the enzyme should be permanently retained in this material.

The catalyst obtained by the in-situ method (synthesis of mesoporous silica in a solution containing the enzyme) follows the leaching trend of previous ones immobilized through this technique [35] where a small percentage of enzyme is released in the first 2 h corresponding to enzyme molecules immobilized on the external surface of the catalyst particles. In this case the leaching was 3% and after this initial period, no more enzyme is detached (Figure 1).

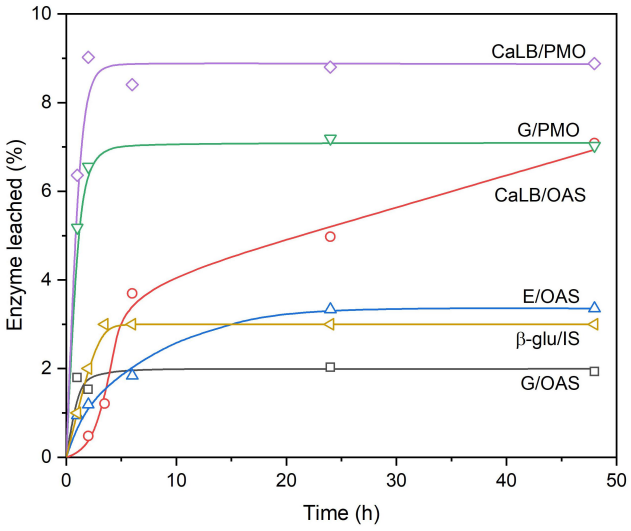


Figure 1. Enzyme leaching from biocatalysts through a 48 h period at pH 6.0 and temperature of 35°C. Symbols correspond to experimental data. Lines are a guide to the eye.

Lipases and esterases: Leaching profiles of the lipases CaLB and G immobilized on octyl amorphous silica and PMO, and esterase E immobilized on octyl amorphous silica were studied. Despite the mild hydrophobicity of ethylene groups, the high density of them in PMO succeeds to provide a strong affinity with lipases thus yielding high enzyme loadings (see Table 1) and succeeding to retain the enzyme. Although initially 6% and 8% are released from G/PMO and CaLB/PMO respectively (presumably weakly linked enzyme molecules), the protein content remains constant for the following 40 h of leaching test, meaning that no more enzyme is detached. This profile is shared by lipase G immobilized on both, OAS and PMO. Despite the strong hydrophobic attraction with octyl groups, lipase CaLB and esterase E immobilized on OAS seemed to be less protected: the wide pore of this material fails to provide confinement enough to enzyme molecules, so they are more loosely bound to the support and thus the enzymes show a slow but continuous release profile along the test.

Two-step oleuropein hydrolysis

Hydrolysis of glucosidic bond with beta-glucosidase

The first strategy tested for enzymatic transformation of oleuropein into hydroxytyrosol was a two-step process based on the cleavage of the

glucosidic bond of oleuropein and further hydrolysis of the aglycon (see Scheme 1).

Oleuropein hydrolysis to obtain the aglycon was catalyzed by immobilized β-glu. Reactions were carried out with pure oleuropein and with a commercial extract from olive leaves containing 20% oleuropein. Pure oleuropein was used just to check primary reaction conditions and HPLC peaks identification. Commercial extract was preferred to perform all the studies since the objective of this work was the use of the agricultural waste as the source of oleuropein. Retention times of the main peaks (oleuropein and hydroxytyrosol) were determined from the pure compounds as described in Methods.

The parameters of the reactions studied were: pH, amount of catalyst, substrate concentration and temperature (Figure 2).

(1) Catalyst concentration and pH
In a first approach, the amount of biocatalyst in the reaction was optimized using 50 mg and 100 mg in the 10 mL reaction volume (5 and 10 mg·mL^{-1}, respectively) at two different pH values. According to results shown in Figure 2a, the increase of enzyme does not affect the rate of oleuropein hydrolysis. What this figure shows is that the pH is a more relevant parameter.

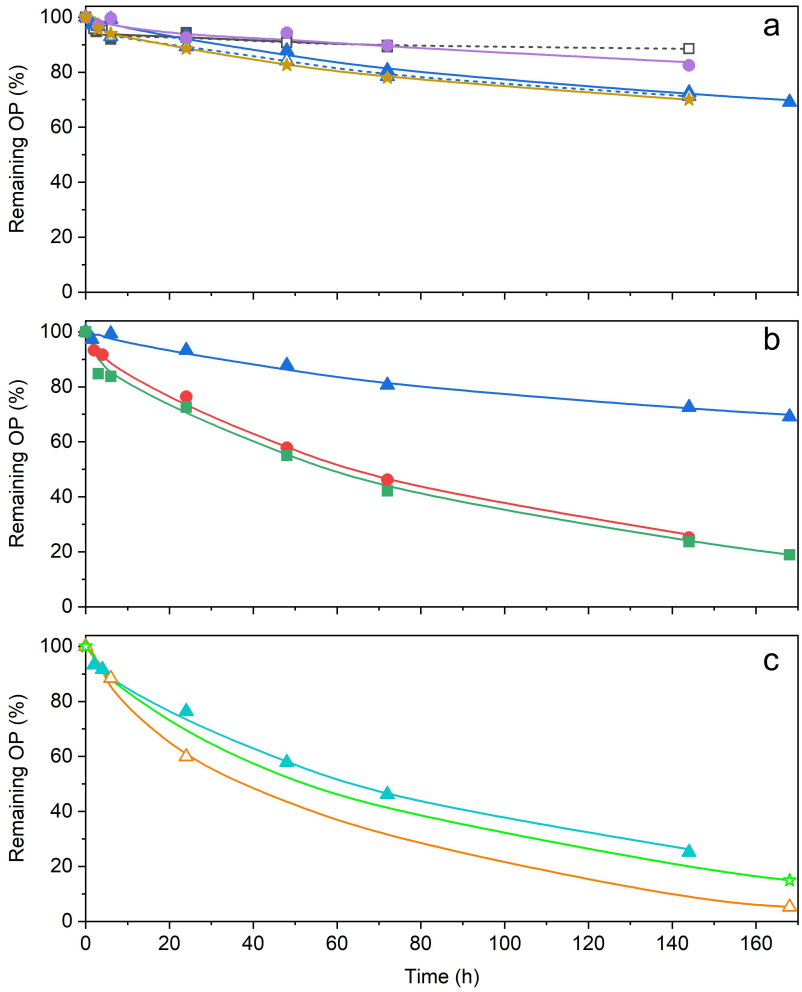


Figure 2. Oleuropein conversion during hydrolysis under different conditions. a) hydrolysis of 20 mM OP solutions at 40°C and pH 5.0 (squares), 6.0 (circles), 6.5 (stars) and 7.0 (triangles) using a biocatalyst ratio of 5 (full symbols) and 10 mg·mL^{-1} (open symbols). b) hydrolysis in solutions with OP concentration of 20 (triangles), 5 (circles) and 2.5 mM (squares) at 40°C and pH 7.0 using 5 mg·mL^{-1} biocatalyst ratio. c) hydrolysis of 5 mM OP solutions using 5 mg·mL^{-1} biocatalyst ratio at pH 7.0 (triangles) and 6.5 (stars) and a temperature of 40 (full symbols) and 50°C (open symbols). Symbols correspond to experimental data. Lines are a guide to the eye.

So, 5 mg·mL^{-1} biocatalyst was selected to catalyze the reaction at pH 5.0, 6.0, 6.5 and 7.0 (Figure 2a), showing that the higher rate of OP hydrolysis was achieved at pH 7.0. However, the reaction rate was still rather slow under these conditions, reaching OP conversion of only ca. 30% after 7 days of reaction.

(2) OP concentration
Since β-glu is inhibited by the glucose released in the reaction, it is of the utmost relevance to know the maximum OP concentration that can be used to avoid this unwished effect. Therefore, a series of reactions with initial OP concentrations between 2.5 and 20 mM were carried out at pH 7.0 with 5 mg·mL^{-1} biocatalyst. The results clearly showed that the limiting step is the inhibition of beta glucosidase by the glucose released in the reaction. As shown in Figure 2b, it was necessary to decrease oleuropein initial concentration in order to avoid the inhibitory effect. Lower concentrations tested (5 and 2.5 mM) were hydrolyzed at the same rate. Since this work has as the ultimate goal the industrial applicability of the method the highest possible concentration which avoids product inhibition, i.e. 5 mM, was chosen for the subsequent tests.

(3) Temperature
All the studies described above were performed at 40°C. Once stated the best reaction conditions at that temperature, the effect of increasing reaction temperature to 50°C was studied at pH 6.5 and 7.0 (Figure 2c). It was found that the best conditions for the hydrolysis of oleuropein, allowing full conversion of OP at the highest rate, were pH 7, temperature 50°C, initial concentration of oleuropein 5 mM and 5 mg·mL^{-1} biocatalyst concentration. Using these conditions, OP was almost totally hydrolyzed within 7 days. The supernatant from the reaction performed under these optimal conditions was separated and studied for the next step: hydrolysis of the oleuropein aglycon to obtain hydroxytyrosol.

Hydroxytyrosol obtention from oleuropein aglycon

Two different approaches were tested, a non-enzymatic one based on thermal treatment, and the other using the esterase LAE6 immobilized on $SBA15NH_2$ in order to cleave the ester bond to obtain hydroxytyrosol [36].

Thermal treatment described by Briante seemed easy and simple: just to increase the temperature of the oleuropein aglycon solution to 60°C at pH 7 [15]. However, the results (Table 2) showed no HT production when the aglycon obtained from 5 mM OP solution (prepared using pure OP) was heated at 60°C, nor 70°C. Nevertheless, when the reaction was performed with a solution of extract from olive leaves, and starting with the same concentration of OP (5 mM), a small amount of HT was obtained upon incubation at 70°C (Table 2). According to these results, it seems likely that some esterase might be present in the extract from leaves.

Once discarded the thermal strategy, an immobilized esterase was used to cleave the ester bond and release the HT (Scheme 2). Giannakopoulou et al. [37] used a two-enzyme system with co-immobilized beta glusosidase and CaLB. Pure oleuropein was used in this experiment instead of the leaves extract in order to reduce impurities that may hinder the HT production. The esterase was assayed at two different steps of the reaction. In the first one, the esterase LAE6/$SBA15NH_2$ was added to the supernatant of pure oleuropein hydrolysis reaction, this is, the aglycon solution. Upon treatment with the enzyme, 0.498 mM HT was obtained as seen in Table 2.

Table 2. Maximum concentration of HT (mM) obtained through different reaction routes starting with 5 mM OP from leaves extract or pure OP. *immobilized esterase LAE6/$SBA15NH_2$.

OP source	Thermal treatment of aglycon		Esterase*	
	60°C	70°C	On aglycon	On OP molecule
Leaves extract	0	0.0077	nd	nd
Pure OP	0	0	0.498	0.588

This yield compares to the reported one from other non-enzymatic routes used in industry to obtain this compound [9]. The strategy of HT synthesis using esterase is not only smarter but also more efficient than just heating.

In the second one, the esterase was added over the whole oleuropein molecule, prior to the split of glucosidic bond. Upon addition of the immobilized esterase LAE6/SBA15NH$_2$ on freshly prepared oleuropein solution (using pure OP from Sigma), 0.588 mM of HT was detected.

From this approach, the direct use of esterase seems to suggest that direct cleavage of ester bond in oleuropein to release hydroxytyrosol would be advantageous.

One-step oleuropein hydrolysis

Enzymatic transformations of OP into HT in one step reactions were carried out starting from olive leaves extract.

Survey of immobilized lipases and esterases for one-step HT obtention

The feasibility of this one-step reaction encourages to survey other hydrolytic enzymes and reaction conditions for this process (Scheme 1). Amongst enzymes with esterasic activity, some commercial lipases were selected to be tested due to their easier availability in larger amounts, as well as an esterase obtained from olive brine. For this study, immobilization of the enzymes on OAS supports were preferred because of their simpler preparation and large pore sizes, which makes them effective regardless the enzyme size.

Biocatalysts were initially tested in a 24 h reaction to identify which lipases were suitable for the obtention of HT using the olive residue extract as substrate (containing small concentrations of HT as seen in Figure 3). Figure 3 shows the HT obtention in these exploratory reactions carried out at room temperature. Noticeably, the absence of enzyme inhibition allowed working with starting concentration of OP 12 mM, i.e. higher than in the reaction with beta glucosidase.

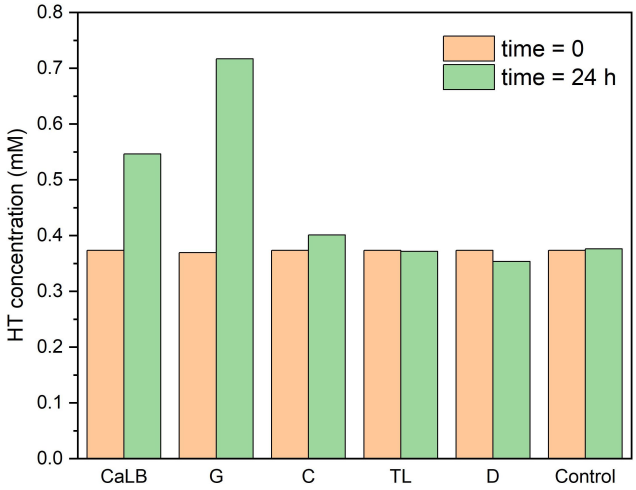


Figure 3. HT obtained with various lipases immobilized on OAS. HT concentration in the OP solution is shown for each biocatalyst before and after 24 h incubation. Control: OP solution without enzyme. Conditions were room temperature, pH 7.0, 12 mM OP and 2 mg·mL^{-1} biocatalyst.

As shown in Figure 3, only Lipases G and CaLB produced a significant amount of HT in the test. These two lipases also showed the highest enzymatic loading in AOS. For these two reasons, they were selected to also be immobilized in PMO and tested in the reaction. Esterase E was not immobilized on PMO because its pore dimensions are not wide enough for the large molecular size of this enzyme (electrophoresis shown in Figure S2 suggests a molecular weight around 60 KDa). The enzyme loadings of all the obtained biocatalysts were determined (Table 1), which enables to calculate the amount of each catalyst necessary to perform all the reactions with the same amount of enzyme.

Study of one-step reaction conditions to obtain HT from OP

Enzyme concentration was the first parameter to study. Figure 4 shows the HT obtention with equal amounts of lipase from CaLB: Lipase CaLB/OAS, 2 mg per mL of reaction suspension, and CaLB/PMO, 2.9 mg per mL of reaction suspension, so that both systems have 0.2 mg of enzyme per mL of suspension. A lower concentration of Lipase CaLB/PMO, 1.4 mg·mL^{-1} was also tested having 0.09 mg of enzyme per mL of suspension. Lipase

G/OAS and G/PMO biocatalysts have equal enzyme loads, 0.09 mg of enzyme per mL of suspension.

With equal enzyme amounts in the reaction systems, OAS-based biocatalysts perform better than their PMO counterparts. Figure 4b shows that a twofold increase of CaLB on PMO is hardly noticeable on HT yield, being this result also close to the obtained with lipase G on PMO (Figure 4a). A possible explanation involves the much larger pore sizes of the OAS supports, enabling substrate and products transport in and out of the particles.

Experiments were carried out to study the effects of parameters like temperature, pH, OP initial concentration and biocatalyst concentration on the HT production catalyzed by immobilized CaLB and Lipase G.

a) Temperature. Figure 5 shows the effect of different temperatures on the kinetics of HT obtention for the lipases G and CaLB biocatalysts, both in OAS supports. Lipase G shows decreased HT obtention with temperature increase and

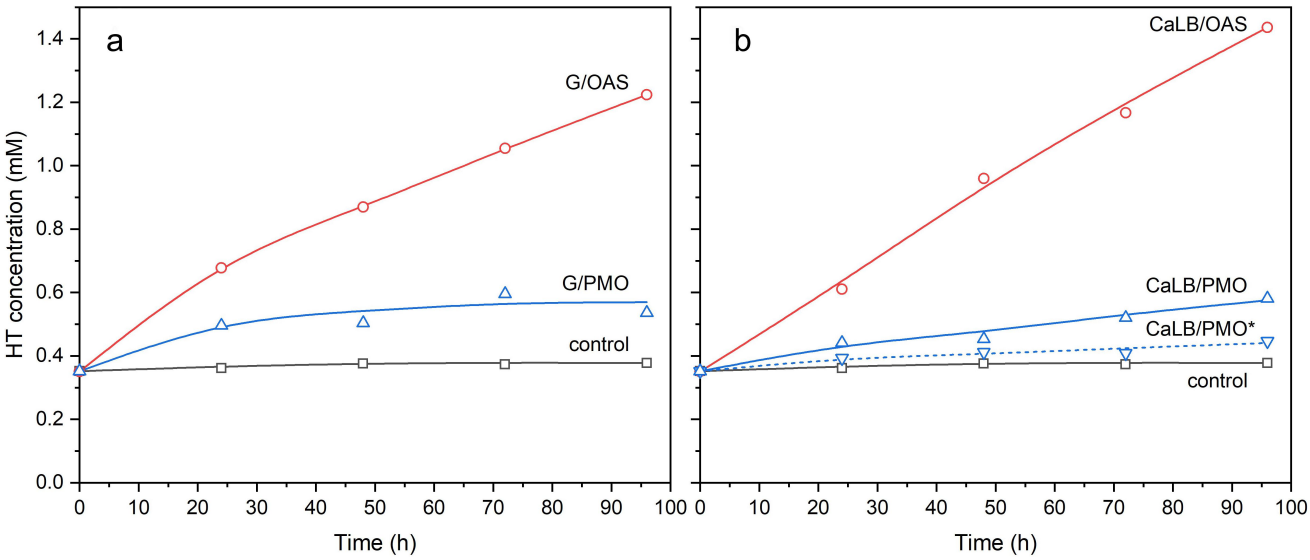


Figure 4. HT obtention using biocatalysts prepared with OAS or PMO supports. (a) Lipase G, 0.09 mg of enzyme per mL of suspension. (b) Lipase CaLB, 0.2 mg of enzyme per mL of suspension (* 0.09 mg of enzyme per mL of suspension). Control: OP solution without enzyme. Conditions were 40°C, pH 7, and 12 mM OP solution. Lines are a guide to the eye.

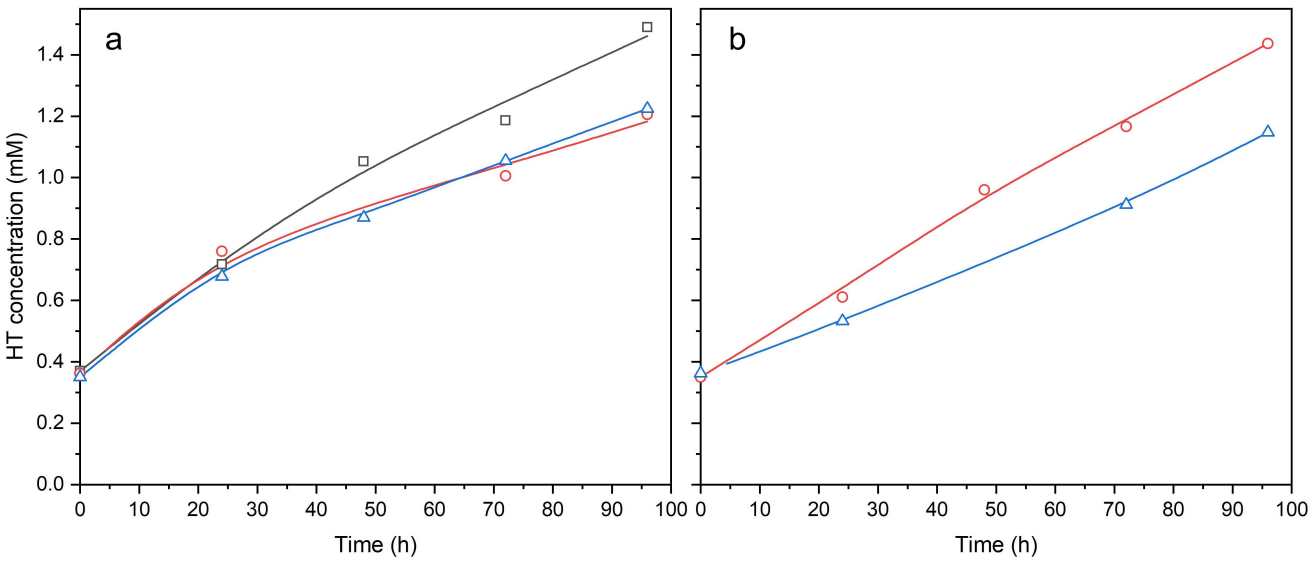


Figure 5. HT obtention at different temperatures using biocatalysts prepared with OAS support. (a) Lipase G. (b) Lipase CaLB. Conditions were pH 7, 12 mM OP, 2 mg·mL^{-1} biocatalyst and temperatures of 30°C (squares), 35°C (circles) and 40°C (triangles). Lines are a guide to the eye.

performed better at 30°C, while lipase CaLB shows the opposite behavior, i.e. increased HT production with increased temperature and performed better at 40°C. A compromise temperature to carry out further reactions was chosen at 35°C as both biocatalysts showed similar performance in terms of HT production.

b) Substrate (OP) and biocatalyst concentration. Figure 6 shows the effect of initial OP concentration and biocatalyst concentration on the rate of HT obtention. The value of each parameter was doubled separately, while maintaining the other one in the lower value.

Figure 4b already provided an example of the effect of higher biocatalyst concentration for the CaLB/PMO biocatalyst, showing increased HT production. In this section, 2 and 4 $mg{\cdot}mL^{-1}$ of the respective OAS catalysts are compared. Increasing biocatalyst concentration showed increased performance with higher HT production, for the same OP concentration of 12 mM.

With a constant amount of 2 $mg{\cdot}mL^{-1}$ catalyst, the concentration of OP was increased from 12 to 25 mM, the increase in reaction rates of HT production not resulting significantly higher. These data suggest that there is not much room to increase the reaction rate so probably these conditions are close to those corresponding to zero order reaction, but still the slopes corresponding to 12 mM OP/4 $mg{\cdot}mL^{-1}$ catalyst are higher than those with 25 mM OP/2 $mg{\cdot}mL^{-1}$ catalyst. Thus, in the conditions tested, the lipase catalysts concentration seems to be the limiting step for HT obtention, the substrate concentration being high enough to approach the maximum rate of enzymatic reaction.

With this one-step enzymatic reaction, the initial OP concentration can be increased up to 5-fold when compared to the two-step reaction with β-glucosidase, where the maximum concentration of OP in the reaction was limited to 5 mM due to the inhibitory effect of glucose as a product of the reaction (Figure 2).

c) pH. Spontaneous OP degradation may occur and it has to be considered. Therefore, the pH of the reaction mixture is a meaningful condition for the hydrolysis of OP. Three pH values were tested for the reaction: 6.0, 7.0 and 8.0. Figure 7 shows the non-enzymatic degradation of OP through an incubation time of 168 h at these pH values. An OP solution at pH 6.0 shows slower OP degradation with respect to solutions at pH 7.0 and 8.0. Table 3 shows that the yield of HT (moles of HT produced per mole of OP fed) for the enzymatic reactions at pH 6 is higher in both cases. This means that the faster degradation of OP in the absence of enzyme at pH 7 and 8 indicates a faster unspecific, undesirable process, particularly at

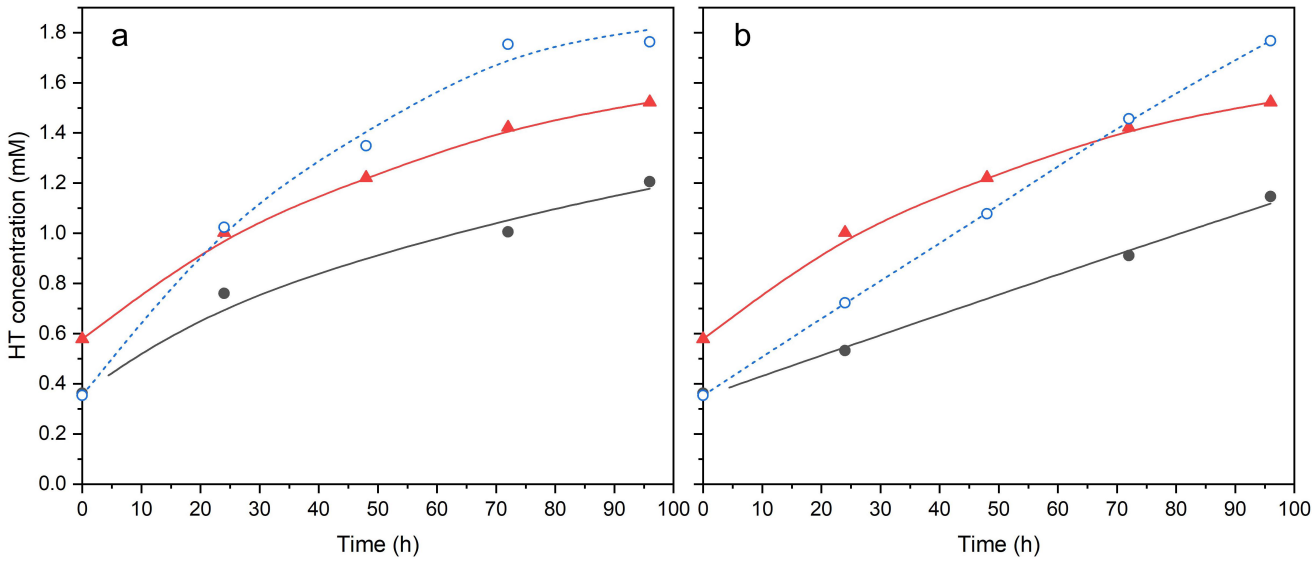


Figure 6. HT obtention with different concentrations of OP and biocatalysts prepared with OAS support. (a) Lipase G. (b) Lipase CaLB. Conditions were pH7, temperature of 35°C, 12 mM (circles) or 25 mM OP (triangles) and 2 $mg{\cdot}mL^{-1}$ (full symbols) or 4 $mg{\cdot}mL^{-1}$ biocatalyst (open symbols). Lines are a guide to the eye.

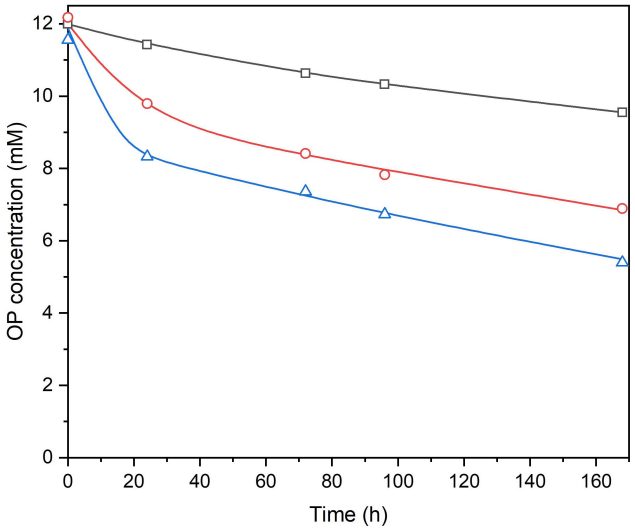


Figure 7. OP degradation in control solutions at different pH values. Conditions were temperature of 35°C, 12 mM OP, no biocatalysts added and pH of 6 (squares), 7 (circles) or 8 (triangles). Lines are a guide to the eye.

Table 3. OP conversion and HT yield after 168 h of enzymatic reactions at different pH values for biocatalysts lipase G and CaLB on OAS. Reaction conditions were: temperature 35°C, initial OP concentration 12 mM and biocatalyst concentration 2 mg·mL^{-1}.

pH	Biocatalyst	OP conversion (%)	HT yield (%)
6.0	Lip G	50.3	14.1
	CaLB	48.1	11.1
7.0	Lip G	58.6	10.5
	CaLB	78.3	10.9
8.0	Lip G	65.2	8.2
	CaLB	89.4	3.3

the highest pH value. And it also means that a lower unspecific hydrolysis of OP (at the lowest pH) keeps higher concentration of this substrate available for the enzyme.

d) Reaction in optimal conditions. The pH was chosen as 6.0 to benefit from the higher yield achieved and both biocatalyst and substrate concentration were doubled. Temperature was fixed at 35°C as previously discussed. 25 mM was the starting concentration of the substrate OP, and the amount of the respective catalysts was 4 mg·mL^{-1}.

Figure 8a shows that HT production with biocatalyst E is very close to that of the control sample. Considering that this enzyme was not necessarily operating at its optimum set of conditions (pH, temperature and concentration) these results are encouraging to determine how much these biocatalysts could be improved through experiments like those performed with biocatalysts G and CaLB.

In accordance with the experiments described above, biocatalysts using OAS as the support for immobilization performed better than their respective PMO counterparts when comparing the concentrations achieved with lipases from the two different sources, as seen on Figures 8b,c. The presence of narrow openings in PMO channels not only limits the accessibility of the enzyme, thus leading to lower enzyme loading in PMO, but also restricts the diffusion of OP, both reasons being most likely responsible for the low yields of OP transformation into HT.

Higher yields of OP hydrolysis and HT obtention were obtained with Lipase G. Interestingly, the shape of HT concentration profile in Figure 8c indicates that the biocatalysts are still active to produce HT after 12 days.

The apparent biocatalyst stability under reaction conditions after several days of incubation is also an important factor for its reusability. The OAS-Lipase G biocatalyst proved to retain its activity for at least 12 days, achieving HT concentration as high as 6.8 mM (1 g·L^{-1}), with a yield of 29% over the OP fed. The results of this reaction surpass those based on the split of glucosidic bond with beta-glucosidase shown above (Table 2) where HT concentration was 0.588 mM after 120 h of incubation. Lipase G/OAS permits to obtain double HT concentration in less than 24 h and the total increase over the 288 h incubation was over elevenfold. The maximum HT concentration obtained of 1 g·L^{-1} is higher than the 0.8 g·L^{-1} reported using an enzymatic extract of *A. niger* [24], a result that was achieved in 2 h but with a non-recoverable catalyst. The same concentration was achieved by the Lipase G/OAS biocatalyst in 192 h and the catalysts were still active close to 100 h after that. It is possible that with increased enzymatic loadings or biocatalyst concentration and an optimal reactor, the production of this biocatalyst will match those values while also being reusable, for example, in a continuous processing production plant. This is to be expected because of the high enzyme retention shown in the leaching tests (Figure 2).

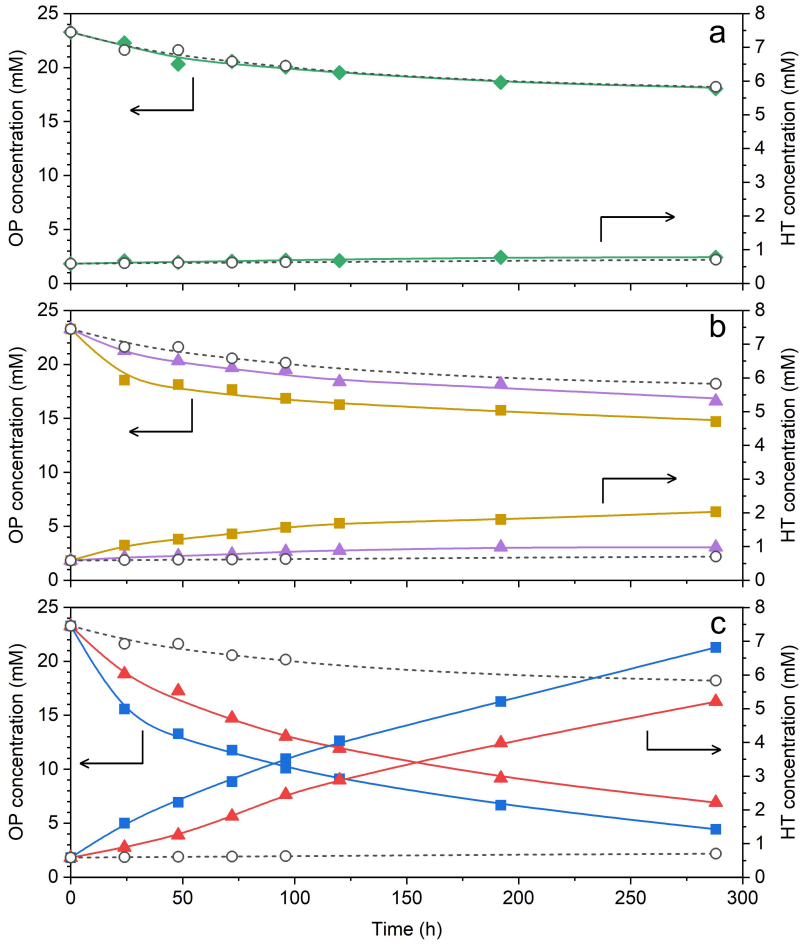


Figure 8. OP conversion and HT production in enzymatic reactions under best performing conditions through a 288 h incubation period using biocatalysts prepared with OAS and PMO supports. (a) Esterase E/OAS (rhombus). (b) Lipases G (squares) and CaLB (triangles) on PMO support. (c) Lipases G (squares) and CaLB (triangles) on OAS support. Conditions were pH 6, temperature of 35°C, 25 mM OP and 4 mg·mL^{-1} biocatalyst. Results of control test (without biocatalyst addition) are shown as open circles and broken lines. Lines are a guide to the eye.

e) One-step versus two-step process. The first approach that we have explored to produce hydroxytyrosol was a two-step process that makes use of beta glucosidase in a first reaction step to catalyze the hydrolysis of oleuropeion to obtain the aglycon, which is further hydrolyzed to HT in a second step. Beta glucosidase undergoes inhibition by glucose, a product of the reaction. Therefore, the initial substrate concentration must be low in order to overcome inhibition by product. In this first approach, oleuropein aglycon is obtained by split of the glucosidic bond catalyzed by beta glucosidase. At pH 7 and 50°C, with initial concentration of oleuropein 5 mM, OP was almost totally hydrolyzed within 7 days using 5 mg·mL^{-1} biocatalyst concentration. The aglycon obtained in the first reaction step was then submitted to a second reaction step in order to hydrolyze the ester bond of the aglycon to yield hydroxytyrosol. Thermal hydrolysis failed when the aglycon was obtained using pure oleuropein in the first reaction step. However, when leaves extract was used as source of OP, a small amount of HT (lower than 0.01 mM) was obtained, which suggests the presence of some esterasic activity in the extract. Therefore, a second approach was explored, namely the enzymatic hydrolysis of the aglycon with an esterase. The supernatant of 5 mM OP total hydrolysis with glucosidase (first step) yielded almost 0.5 mM HT (in the second step) using esterase LAE6/SBA15NH2, which is around 10% yield. A similar yield (around 0.6 mM) was obtained when the reaction of this esterase was performed directly on pure OP.

As mentioned above, the inhibition of beta glucosidase by glucose requires the use of a low initial concentration of OP, which unavoidably leads to obtaining HT in very low quantities. These findings led us to the explore the second approach, where the use of enzymes with esterasic activity permits both performing a one-step reaction, and starting it with a higher substrate concentration. The split of the ester bond of OP molecule yields directly HT, so the handicap of beta glucosidase inhibition by glucose does not apply here. 25 mM OP from leaves extract can be used as starting concentration. Amongst the esterase and lipases tested and immobilized by two different approaches, the lipase G immobilized on octyl amorphous silica by hydrophobic interactions turned out to be the best catalyst for this reaction: 4 $mg{\cdot}ml^{-1}$ biocatalyst yielded 6.8 mM HT after 12 days in a one-step reaction carried out at 35°C and pH 6.0.

So, an in-depth study of the issue of the transformation of OP into HT has led us to develop increasingly simpler and more efficient reaction strategies. 6.8 mM HT (which is around 30% transformation) was obtained through one-step reaction catalyzed by lipase G, compared to 0.5 mM HT (around 5% transformation) obtained with the two-step approach using beta glucosidase and an esterase in two consecutive reactions.

Conclusions

Transformation of OP into HT was addressed by two strategies with different enzymes. 5 $mg{\cdot}mL^{-1}$ β-glucosidase immobilized *in situ* in siliceous ordered mesoporous material catalyzed the hydrolysis of 5 mM OP at pH 7.0 and 50°C to give the OP aglycon. Further enzymatic hydrolysis of the aglycon with an esterase immobilized on $SBA15NH_2$ produced close to 0.5 mM HT. Enzymatic one-step reaction to obtain hydroxytyrosol from oleuropein from an agricultural waste has been successfully performed with 4 $mg{\cdot}mL^{-1}$ lipase from *Penicillium camemberti* immobilized on octyl silica to catalyze the hydrolysis of 25 mM OP at 35°C and pH 6.0. In these conditions 1 $g{\cdot}L^{-1}$ of HT was easily obtained. These results are promising in the context of the already existing HT extraction processes, which would benefit from the increased availability of the compound before extraction, potentially lowering the cost per kg of HT. For this reason, further optimization of the supports in terms of enzyme loading and leaching reduction, reaction conditions and biocatalyst durability may be researched to provide a cost-effective process to recover this very high added value product from an agricultural residue.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

Gabriel García-Molina: Investigation, Formal analysis, Writing – Original Draft. Eduard Peters: Investigation, Formal analysis, Writing – Original Draft. Rosa Palmeri: Methodology,

Investigation, Resources. Yaregal Awoke: Methodology, validation. Carlos Márquez-Álvarez: Methodology, Validation, Writing – Review & Editing. Rosa M. Blanco: Conceptualization, Writing – Review & Editing, supervision.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

Ethical approvals

No ethics approval was required for this study as it involved no human participants or animals

Highlights

- Olive leaves residues are used to obtain high added value antioxidant HT from OP
- Different enzymes, supports and immobilization methods were used to catalyze the biotransformation
- Two steps reaction: β-glucosidase splits glucosidic bond, and further hydrolysis of aglycon
- One-step: lipases or esterases hydrolyze the ester bond to release hydroxytyrosol.
- 6.8 mM HT obtained from 25 mM OP in one step catalyzed by lipase from *P. camembertii* in octyl-silica

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