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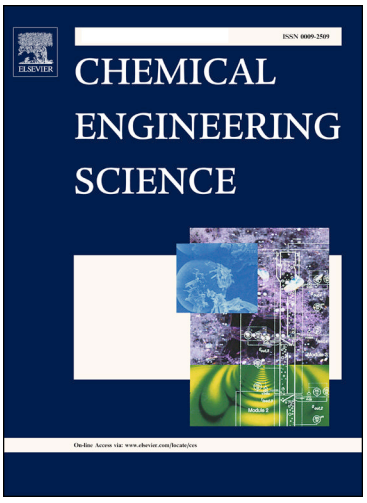

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Biorefinery of olive leaves to produce dry oleuropein aglycone: use of homemade ceramic capillary biocatalytic membranes in a multiphase system

Giuseppe Ranieri[1], Rosalinda Mazzei[1*], Teresa Poerio[1*], Fabio Bazzarelli[1], Zhentao Wu[2], Kang Li [3,4], Lidietta Giorno

[1]Institute on Membrane Technology ITM-CNR, Via P. Bucci CUBO 17C, 87036 Rende (CS) Italy

[2]Aston Institute of Materials Research, School of Engineering and Applied Science, Aston University, Birmingham, B4 7ET, UK

[3]Barrer Centre, Imperial College London, South Kensington, SW7 2AZ, London, UK

[4]Department of Chemical Engineering, Imperial College London, South Kensington, SW7 2AZ, London, UK

* Corresponding author; tel: +39 0984 492076: fax: +39 0984 49

e-mail: r.mazzei@itm.cnr.it ; t.poerio@itm.cnr.it

Abstract

Oleuropein aglycone is an important antioxidant compound produced during oleuropein hydrolysis, not yet commercially available. Its production from renewable material by green processes is a challenge because it permits waste re-use and low environmental impact. In this work, homemade asymmetric capillary ceramic membranes were used to develop biocatalytic membranes, which were further used to produce oleuropein aglycone from olive leaves and/or commercial oleuropein. Results indicated that the biocatalytic system (containing covalently immobilized β-glucosidase) promotes the hydrolysis of oleuropein in both monophase and multiphase processes. Furthermore, the multiphase biocatalytic system enables the extraction of the hydrophobic oleuropein aglycone in an organic phase, before its rearrangement in water. This was achieved by the production, of an unstable water-in-oil emulsion (permeate side), on the basis of membrane emulsification process. The intensified biocatalytic/extractor system allowed taking shelter the hydrophobic compound in the organic phase with good efficiency (90%), protecting it from rearrangement.

Keywords: oleuropein aglycone, 3,4-DHPEA-EA, multiphase membrane reactor, capillary ceramic membranes, phytotherapic production, waste valorization, green technology

1. Introduction

In the last century, the use of natural medicine has expanded and many researchers focused their work on active components production/extraction from vegetal material. These components are known as plant secondary metabolites [1] and possess impressing antioxidant, antimicrobial, antiviral and anti-inflammatory properties [2-4]. Phytochemicals in fruits and vegetables act synergistically and additively to provide potential health benefits against chronic diseases by inhibiting the harmful effects of free radicals [2, 4-6]. The traditional Mediterranean diet is characterized by high consumption of foods of plant origin, relatively low consumption of red meat, and high consumption of olive oil. Olive oil has been extensively reported in studies to contain

physiologically active polyphenols, with oleuropein aglycone as one of the most important [7, 8]. Olive leaves are also rich in polyphenols, especially in oleuropein, rutin, verbacoside, apigenin-7-glucoside and luteolin-7-glucoside [9], and as a result they can become a renewable material from which important non commercially available phytotherapics can be extracted/produced. The concentration of polyphenolic compounds in olive leaves changes (60-90 mg/g dry weight), depending on the quality, origin and variety [10] of the plant material.

Oleuropein is the most abundant polyphenol in olive fruits and leaves, which has been used in several medical treatments [9], while its hydrolysis products (particularly the isomer of oleuropein aglycone, 3,4 DHPEA-EA) are abundant in olive oil. In olives and olive leaves, this compound is hydrolyzed by the action of β-glucosidase, forming oleuropein aglycone and a molecule of glucose in the first reaction step. It has been demonstrated that the oleuropein aglycone has various types of potential applications, such as an antimicrobial agent in some fairly common diseases of olive trees [5] or in pharmaceutical sector as antioxidant [6, 8, 9, 11, 12], as bactericidal and bacteriostatic agent [13] as platelet aggregation inhibition component [14], as anticancer and [15], antiviral compound [16, 17].

More recently, oleuropein aglycone has gained increasing interest because of its beneficial effect on neuroprotection [18] in neurodegenerative disease and reduction of acute inflammation (carrageenan-induced pleurisy) [19]. The main effect is given by its prominent hydrophobic character, which allows it to interact directly with biological membranes [20] and to increase its local concentration [16], with important consequences on its beneficial pharmacological effect on cells.

In addition, oleuropein derivatives are involved in the production of compounds with organoleptic properties, which can influence bitterness of olive oil [21] and olive-based foods. Therefore, oleuropein aglycone, once produced, could play an important role in the production of olive oil and olive food derivatives.

Despite the reported importance of the oleuropein aglycone, this molecule is still commercially unavailable, mainly due to its poor stability in water. Typically, about 24 h [13] after its production, oleuropein aglycone is rearranged into water soluble compounds and lost preferred properties aforementioned. This is why, instead of being in olive mill wastewater, it is normally present in olive oil, and together with hydroxytyrosol, yield in the high antioxidant properties of olive oil [22]. In this scenario, a multiphase system to efficiently sequestrate the molecule before its rearrangement in water, after its production, is mandatory. This aim will be achieved in this study integrating a biocatalytic membrane reactor with a membrane emulsification process [23, 24].

The system allows the hydrolysis of oleuropein in the aqueous phase within the biocatalytic membrane. The aqueous phase, containing the products, passes on the other side of the membrane, where an organic phase is recirculated. In the permeate side, on the basis of membrane emulsification process a water-in-oil emulsion is produced, which permits the extraction of the hydrophobic oleuropein aglycone. This will promote the production/extraction of the oleuropein aglycone in one step, before its rearrangement in water phase. The subsequent solvent removal will permit to obtain a solid oleuropein aglycone to be used for formulated products in different applications.

Furthermore, in the logic to develop a green chemistry approach, both pure oleuropein and olive leaves extract (containing oleuropein) will be fed to the biocatalytic system to promote the production of oleuropein aglycone from waste and renewable material.

Polymeric membranes were the most commonly used to develop biocatalytic membrane reactors [23]. More particularly, asymmetric capillary polymeric membranes possess a number of special advantages, especially a very large membrane surface area per unit volume and consequently a high surface area available for enzyme immobilization. Although polymeric membranes have been widely used for the development of biocatalytic membrane reactors, their organic nature could be a limitation to chemical stability, when such membranes are in contact with organic solvents that are commonly used for membrane functionalization, as well as in multiphase membrane reactors. In

contrast, inorganic membranes are able to overcome these drawbacks thanks to their outstanding chemical, thermal and mechanical robustness. Significant efforts have recently been devoted to the preparation of ceramic capillary membranes with the same performance of the polymeric ones [25], with to date no applications in a multiphase bioreactor or biocatalytic membrane reported.

In the present work, home-made ceramic capillary membranes with an asymmetric structure were prepared and tested, and for the first time used in a multiphase membrane bioreactor for the continuous production of oleuropein aglycone. In addition to proving the feasibility of using such membranes for both monophase and multiphase biocatalytic membrane reactors, this study will focus on the efficient recovery of oleuropein aglycone in a multiphase reactor, well before its rearrangement.

2. Materials and Methods

2.1 Chemicals

Aluminium oxide powders: 0.01 µm (gamma–alpha, 99.98% metal basis, surface area 100 m^2/g), 0.05 µm (gamma–alpha, 99.5% metal basis, surface area 32–40 m^2/g), 0.3 µm (gamma–alpha, 99.9% metal basis, surface area 15 m^2/g), and 1 µm (alpha, 99.9% metal basis, surface area 6–8 m^2/g), were purchased from Alfa Aesar (a Johnson Matthey company, London, UK) and were used without any treatment. Polyethersulfone (Radal A300, Ameco Performance, Houston, FL, USA), N-methyl-2-pyrrolidone (HPLC grade, Rathbone, London, UK) and Arlacel P135 (Polyethyleneglycol30-dipolyhydroxystearate, Uniqema, Paterson, NJ, USA) were used as binder, solvent and additive, respectively. Tap water and de-ionized water were used as the external and internal coagulants, respectively, during membrane fabrication. Absolute Ethanol (purchased from VWR), (3-Aminopropyl) triethoxysilane 99% (APTES) and Glutaraldehyde solution (both purchased from Sigma-Aldrich) were used for the functionalization of the alumina membrane. Ninhydrin and Glycine (purchased from Sigma-Aldrich) were used as reagent and standard, respectively, for the characterization of functionalized membranes. Sodium dihydrogen phosphate

(NaH_2PO_4) and disodium hydrogen phosphate anhydrous (Na_2HPO_4) (purchased from Sigma-Aldrich) were used to prepare phosphate buffer solution at pH 6. β-glucosidase from Almond (chromatographically purified lyophilized powder, 10-30 U/mg) was obtained from Sigma–Aldrich. Oleuropein was purchased from Extrasynthese (France). Olive leaves extract was kindly provided by the "Centro di ricerca per l'olivicoltura e l'industria olearia, Rende Italy (CREA-OLI)". Before its use, it was pre-treated by ultrafiltration through 30 kDa capillary membranes in order to remove suspended impurities. Centrifugal concentrators Vivaspin 2 (Sartorius Stedim) were used to separate the β-glucosidase from reaction mixture in batch system reactor. Ethyl acetate, octanol, cyclohexane, squalene, toluene, dichloromethane (purchased from Sigma-Aldrich) were used as solvents for the extraction of the reaction product. Acetonitrile and o-phosphoric acid for HPLC mobile phase preparation were purchased from VWR and Sigma–Aldrich, respectively. NaOH and NaOCl (purchased from Carlo Erba, Milan, Italy) were used for the preparation of the membrane cleaning solutions.

2.2 Analytical methods

BCA assay kit was used to evaluate protein concentration. HPLC was used to measure oleuropein and oleuropein aglycone concentrations by adapting a literature method already reported by Mazzei et al. [26]. Briefly, the mobile phase (a mixture of acetonitrile and water (21:79) acidified with o-phosphoric acid up to pH=3) was pumped into a reverse silica Adsorbosphere XL C18 column, 250–4.6 mm, 5 µm (Grace). The flow rate and pressure were 1.2 ml/min and 145 (±4) bar, respectively. The compounds of interest were detected at 280 nm by sample injection of 5 µl.

To measure the amount of amino groups grafted on the functionalized membrane, the ninhydrin method [4] was used. When ninhydrin reacted with grafted amino groups a purple colored compound (Ruhemann's purple) is formed in solution. The absorbance measured at 570 nm of the colored solution is proportional to grafted amino groups. The standard used for the calibration curve is glycine (correlation coefficient between absorbance and concentration: 4000).

2.3 Membrane functionalization and biofuctionalization

Alumina hollow fibre membranes were prepared by a combined phase inversion and sintering technique, which has been used in one of our previous research 27. Briefly, Arlacel P135 (1.3 wt%) was first dissolved in NMP, before adding the alumina powders (58.7 wt%) at a ratio of 1:2:7 (0.01 µm:0.05 µm:1 µm). The formed mixture was rolled/milled with 20 mm milling balls for 48 h, prior to the addition of PESf (6.1 wt%), followed by another ball milling of 48 h. The suspension was then degassed thoroughly to remove air bubbles, transferred to a 200 ml stainless steel syringe controlled by a high pressure syringe pump, and extruded through a tube-in-orifice spinneret at 7 ml/min into a water bath, with an air-gap of 15 cm. Meanwhile, deionized water (10 ml/min) as the internal coagulant was controlled by a second syringe pump and flowed through the central bore of the spinneret. The formed precursor fibres were kept in the water bath for 24-48 h, before being straightened, dried, and calcined in static air at 1450-1500 °C for 4 h to yield ceramic hollow fibre membranes. The porosity of the prepared membrane is 40-50 %".The prepared membranes have an asymmetric structure with a thin sponge-like layer on the shell side and a finger-like voids layer on the lumen side. The membrane surface area used was calculated at 10.9 (±0.7) cm^2, while the membrane void volume, which represents the membrane reactor volume, was 0.18 (±0.03) cm^3. For the membranes functionalization two alumina capillary membranes with internal and external diameter of 0.9 mm and 1.8 mm, respectively, were housed in Pyrex module of 100 mm long. A peristaltic pump feeds the reagent for functionalization or biofunctionalization and two pressure gauges measure inlet and outlet pressures. Figure 1 schematizes the steps employed for membrane functionalization and biofunctionalization.

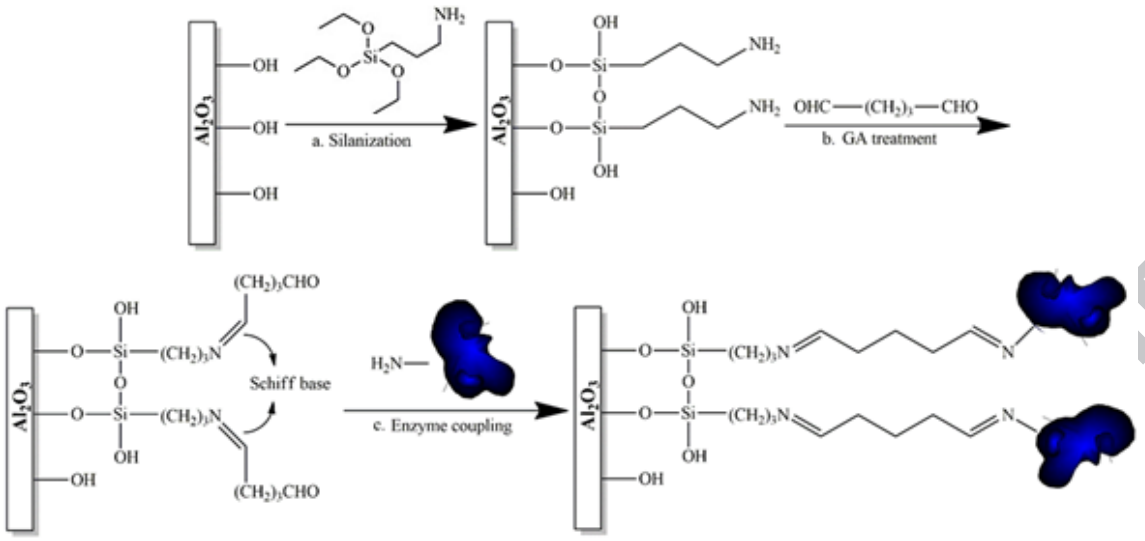


Fig. 1. Scheme of ceramic capillary membranes functionalization and biofunctionalization

The membrane was firstly treated with APTES (silanization step) and then with GA, in order to create aldehydes groups on membrane surface being able to bind the enzyme The percentage of glutaraldehyde (10%), selected among the different percentages tested [27], permitted to immobilize an enzyme amount which gives the highest enzyme activity. Covalent binding occurs through formation of a Schiff base between aldehydic terminal group of glutaraldehyde and amino group of silane. In the last step, the β-glucosidase covalent immobilization was carried out by feeding the enzymatic solution (50 mM phosphate buffer pH 6.5) to the membrane, at room temperature, along the lumen circuit with an axial velocity of 0.033 m/s and a transmembrane pressure of 0.05 bar for 16 h. Membranes were then rinsed with buffers at different pH values (5.5, 6.5, 8.5) (washing solution) to remove any reversibly adsorbed enzyme.

The amount of immobilized protein was determined by mass balance according to the following equation:

$$C_i.Vi = C_f V_f + C_{ws} V_{ws} + m \qquad (1)$$

where *m* indicates the immobilized protein mass in the membrane, *C* and *V* represent the concentration and volume, respectively; the subscripts *i*, *f* and *ws* indicate the initial, final, and washing solutions, respectively. The protein concentration was measured by BCA kit as reported in the section analytical methods.

The amount of immobilized enzyme as a function of the initial protein concentration was also studied, aiming at optimizing the initial protein concentration needed. The initial β-glucosidase concentrations used as feed solutions were: 0.05, 0.10, 0.15, 0.20 and 0.40 mg/ml.

2.4 Monophasic and multiphasic biocatalytic membrane reactors

The hydrolysis reaction was carried out in the monophasic and in multiphasic biocatalytic membrane reactors (Fig. 2), feeding the substrate from shell to lumen. Samples were taken from retentate and permeate as a function of time to determine the presence/amount of the oleuropein, oleuropein aglycon by HPLC, and glucose. This last one was quantified by glucose assay kit.

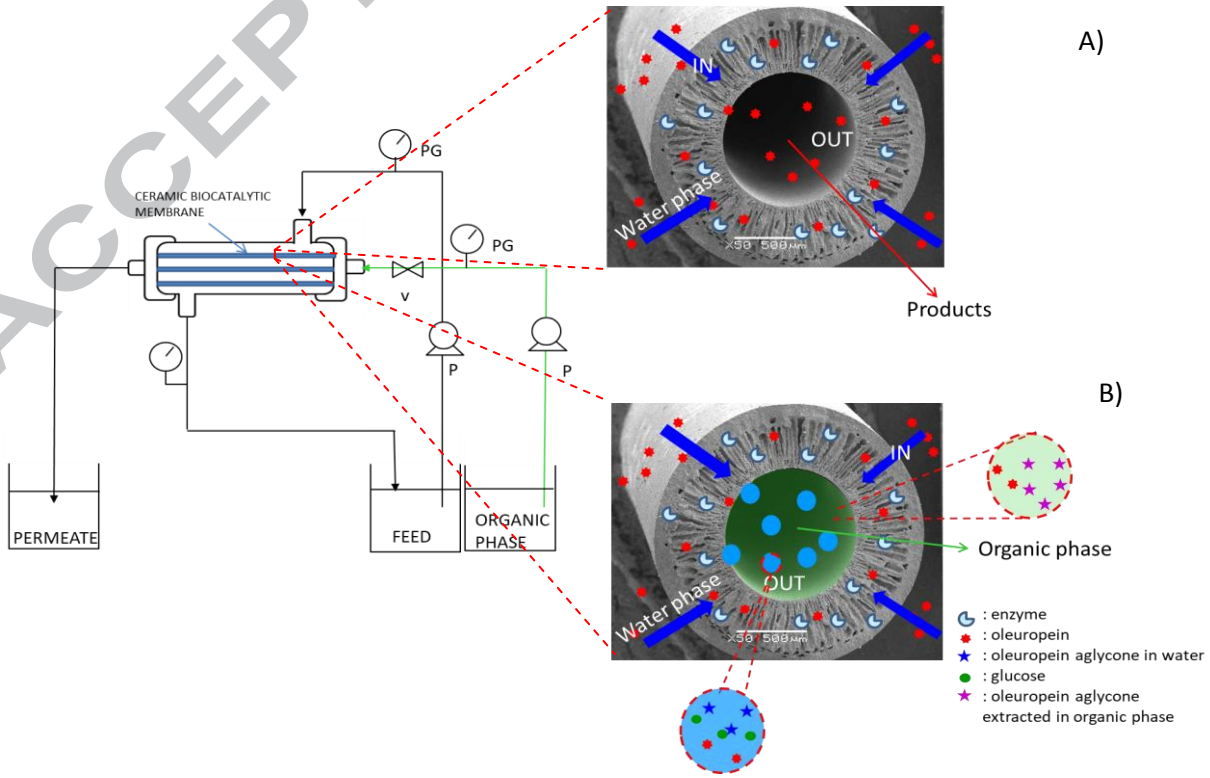


Fig 2. Schematic representation of the monophasic and multiphasic BMR with immobilized β-glucosidase. PG: pressure gauges, P: peristaltic pump, v: valve A) monophase bioacatalytic membrane reactor, B) multiphase biocatalytic membrane reactor

The main difference between the two reactors is that in the multiphase membrane system a selected organic phase is continuously flowing along the lumen side of membranes generating a permeate stream consisting of a water-in-oil emulsion.

When the β-glucosidase reaction was carried out in the multiphase system the permeate flow rate of the water phase was calculated measuring the water volume in the permeate as a function of time by a cylinder. This is possible since the unstable emulsion produced instantaneously separates from the organic phase once collected. The feed volume decrease is also measured as a function of time with a cylinder.In addition to the catalytic/separation activity in monophase reactor, membranes in the multiphase system enable the dispersion of the aqueous phase (containing the products and the unreacted substrate) in the organic phase, which is recirculated on the other side of the membrane on the basis of a membrane emulsification process.

In this way the feed containing the substrate passes through the biocatalytic membrane, where the reaction occurred, then, the products and the unreacted substrate pass on the other side of the membrane, where the unstable water-in oil emulsion was formed. Once collected, the emulsion spontaneously separates permitting to recover the different phases, containing the different compounds extracted between them, on the basis of their partition coefficient.

The biocatalytic membrane reactors were fed with solutions containing commercial oleuropein or extract from olive leaves containing the same substrate (2.5 mM oleuropein, axial velocity of 4.2^{-} m/s, pressure of 0.3 bar). In particular, olive leaves extracts belonging to three different cultivars (Ottobratica, Sinopolese, Ciciariello) were analyzed in order to establish which of them contained the highest oleuropein amount. The harvesting period of olive leaves used for the extraction is July

2014. Before using it as the feed, leaves extract solution was ultrafiltered (30 kDa polysulphone membranes) to eliminate impurities and appropriately adjusted in pH (6.5) and oleuropein concentration (2.5 mM).

During the reaction, the biocatalytic membrane module was kept at 25 °C by thermostatic baths.

The reaction rate in the reactors was derived from the balance equation:

IN-OUT+PROD= ACC (2)

where IN and OUT take into the account the mass of substrate that enters and leave the system, PROD and ACC are the production and the accumulation of targeted product in the same system.

For the mentioned reactors it becomes:

$$(FC)_{IN} - (FC)_{OUT} + (v_r V) = \frac{dVC}{dt} \qquad (3)$$

where F is the flow rate (volume/time), C is the substrate concentration (mass/volume); vr is the volumetric reaction rate (mass/time volume); V is the reaction volume; t is the time.

In these systems, the substrate flow (IN and OUT) occurs by convection and the reaction takes place within the asymmetric porous membrane level, which represents the reactor volume. At the steady state each membrane micropore works as a continuous stirred microreactor, so the overall membrane matrix can be assimilated to a continuous stirred tank reactor (CSTR) working at the steady state.

In this system, the term ACC = 0, so the equation number 3 becomes:

$$(FC)_{IN} - (FC)_{OUT} + (v_r V) = 0 \qquad (4)$$

And the reaction rate is calculated as follows:

$$v_r = \frac{F(C_f - C_p)}{V} \qquad (5)$$

For the stirred tank reactor the flow term is zero and production is equal to accumulation, so the reaction rate is calculated as time variant concentration (C) from the linear section of the curve C vs t.

$$v_r = \frac{dC}{dt} \qquad (6)$$

After the reaction, the biocatalytic membranes were completely regenerated, removing the attached enzyme, using the following developed method: 2% (v/v) of detergent solution, containing 20 wt. % of NaOH, 2.5 wt. % of NaOCl and 77.5 wt. % of water, was pressed through the membrane with a transmembrane pressure of 0.4 bar for 1 h. Membranes were then rinsed several times with deionized water, before next use.

2.4.1 Selection of organic solvents for multiphase membrane reactor

A specific requirement of the solvents to be used as extracting agent in the multiphase system is high oleuropein aglycone extraction, in order to avoid its rearrangement in water. Then, with the aim to select the best organic solvent, the partition coefficient at 25°C of oleuropein aglycone between water and organic phase was determined as follows. An aqueous solution of oleuropein aglycone with an initial concentration of 20 mg L^{-1} was emulsified with ethyl acetate, by a rotor-stator homogenizer (10,000 rpm for 1 minute). The concentration of oleuropein aglycone after the single extraction, both in the aqueous phase and in organic phase, was measured by HPLC as shown in paragraph 2.2. Partition coefficient was calculated by the equilibrium concentration ratio of the oleuropein aglycone (A) in the organic ($[A]_o$) and aqueous phases ($[A]_w$) according to the following equation 7:

$$K_p = \frac{[A]_O}{[A]_W} \qquad (7)$$

Furthermore, degree of extraction E was evaluated as follows (equation 8):

$$E = \frac{(n_A)_O}{(n_A)_W} \qquad (8)$$

where $(n_A)_W$ are moles of oleuropein aglycone in the aqueous phase and $(n_A)_O$ are the moles in the organic phase at equilibrium.

If V_W and V_O are the aqueous and organic phase volumes, respectively, then (equation 9):

$$E = \frac{(n_A)_O}{(n_A)_W + (n_A)_O} = \frac{[A]_O V_O}{[A]_w V_W + [A]_O V_O} = \frac{\frac{[A]_O}{[A]_w}}{\frac{[A]_O}{[A]_w} + \frac{V_W}{V_O}} \quad (9)$$

2.5 Stirred tank reactor

The free enzyme performance was evaluated in a stirred tank reactor (STR) using the following procedure: oleuropein hydrolysis reaction was carried out in 25 ml of total volume, with a reaction mixture composed by 23.5 ml of substrate solution and 1.5 ml of β-glucosidase solution (0.13 mg/ml). The reaction was carried out at 25°C. Both enzymatic and oleuropein solutions were prepared by dissolving their respective lyophilized powders in 50 mM phosphate buffer pH 6.5. In some experiments, glucose was added in the initial reaction mixture in order to monitor its effect as enzyme inhibitor. The initial oleuropein concentrations tested were 0.63, 1.25, and 2.50 mM. For all the mentioned initial substrate concentration the reaction was carried out in absence of glucose (inhibitor) and when its concentration was 1.25 and 2.50 mM, respectively (Supporting information). Samples of reaction mixture were taken as a function of time each 30 seconds. In order to stop the reaction, by enzyme denaturation, and to remove the enzyme from the reaction mixture, the sample was first kept at 100°C and then filtered through centrifugal concentrators Vivaspin 2 (Sartorius).

3. Results and discussion

3.1 Membrane functionalization and biofunctionalization

The membrane functionalization and biofunctionalization is a crucial step to guarantee good performance in the membrane bioreactor. The amount of the amino groups per unit membrane surface generated after the functionalization process was 1.26×10^{-3} ($\pm1.56\times10^{-4}$) mmol/cm^2. The initial water permeability of native membranes decreased by about 37% after the silanization process, and by 47% after the treatment with glutaraldehyde (10 %).

Besides, prior to enzyme immobilization on functionalized membrane, blank experiments were carried out, ultrafiltrating enzyme solution through native membranes, to exclude immobilization due to enzyme adsorption or entrapment. Mass balance according to equation 1, as well as the completely restored permeability (507 (±20) L/hm^{2}bar) demonstrated the absence of enzyme adsorption on functionalized membrane.

Different concentrations of enzyme solution (0.05, 0.1, 0.2, 0.4 mg/ml) were used to biofunctionalize the membranes resulting in 0.9, 2.2, 5, and 7.8 mg of immobilized enzyme, respectively. These results indicated that the amount of immobilized enzyme increases by increasing the concentration of the initial enzyme solution. But, as it can be seen in Fig. 3, the increasing of oleuropein conversion is observed until an initial enzyme concentration of 0.2 mg/ml is used with a corresponding immobilized enzyme of 5 mg.

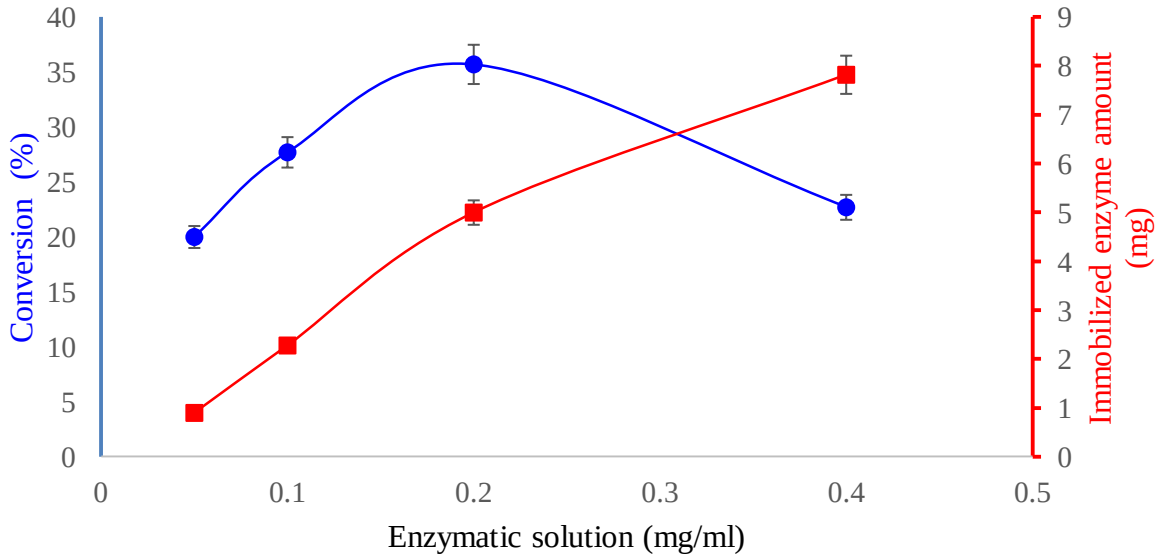


Fig. 3. Amount of immobilized enzyme and oleuropein conversion as a function of initial concentration of enzymatic solution.

Indeed, when the membrane with a higher amount of immobilized enzyme was used, the conversion decreased from 36 (±5) % to 22 (±5) %. This was probably due to enzymatic aggregation on the membrane surface (crowding phenomenon [28]), which impairs the enzymatic activity, as observed

when the same membrane was used to immobilize lipase [27], or immobilizing lipase, protein G and Albumin on polymeric membranes [28]. The conversion achieved is in line with literature, where polymeric membranes and enzyme physical entrapment was used[26].

Applying the membrane cleaning procedure to remove the enzyme, reported in Materials and Methods, the initial water permeability (507(±20) L/hm2bar) was completely restored, also after 6 different enzyme immobilization cycles. This will permit the re-use of the membrane during continuous production process in case of enzyme denaturation.

3.2 Performance of monophasic biocatalytic membrane reactor

Hydrolysis reaction of oleuropein was performed by using biocatalytic membranes loaded with different amounts of enzyme, as above mentioned, and by using pure oleuropein solution (2.5 mM) or olive leaves extracts containing oleuropein as the feed. A constant permeate flux is observed (1.50 L/hm^2, operative conditions: 0.3 bar, 0.3 ml/min, 25°C), confirming that substrate does not interact with the membrane itself, since it can freely pass through the biocatalytic membrane.

The olive leaves extract from the cultivar "Ciciariello" was used as feed solution since, in comparison with the other cultivars (Sinopolese, Ottobratica), it contains the highest amount of oleuropein (2.85 mg/ml). In this extract other polyphenols are present, which do not alter the catalytic action of β-glucosidase towards oleuropein, since the enzyme is highly specific for its substrate, as previously demonstrated by Conidi et al. [29]. Same constant permeate flux as a function of time (1.50 L/hm^2, operative conditions: 0.3 bar, 0.3 ml/min, 25°C), was also observed when ultrafiltered olive leaves extract was fed to the biocatalytic system, demonstrating the little interaction between the feed and the membrane.

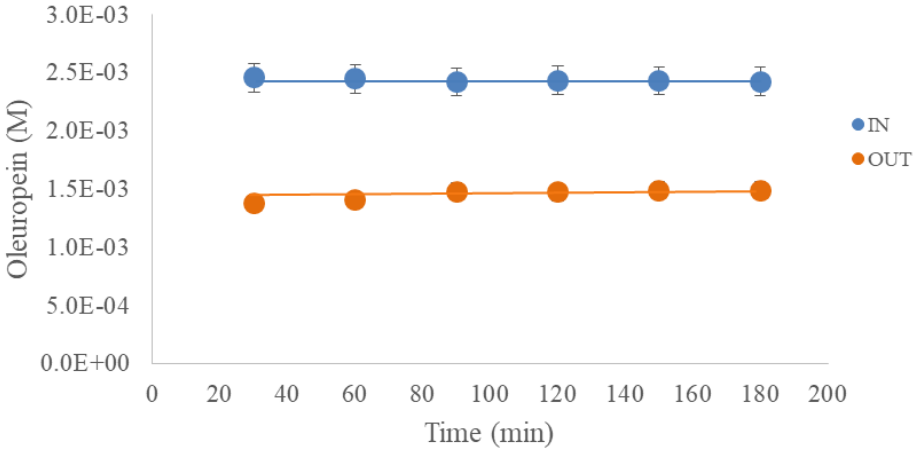
2.5 mM of oleuropein solution was fed to the reactor, which passed from shell side to lumen with an axial velocity of 4.2×10^{-3} m/s and a permeate flow rate of 0.3 ml/min. Here, the substrate met the immobilized biocatalyst and hydrolysis reaction occurred. Aglycone, glucose (reaction products) and unreacted oleuropein were recovered in the lumen circuit of the system. Same operation mode

and operative conditions were then repeated by changing the feed of the reaction with olive leaves extract. Also in this last case, the oleuropein initial concentration was set to 2.5 mM. Any product was detected in the feed and in the retentate collected as a function of time, confirming that it is produced and removed at each passage through the membrane.

A constant substrate concentration profile (Fig. 4a) that enters into the biocatalytic system (IN) and the one unconverted and recovered into the permeate (OUT) is observed. This demonstrated that the system is a continuous system, characterized from time invariant conditions at the steady-state.

The reaction mixture (reagent and products) is continuously removed from the reaction site and no accumulation occurs. So, the reaction rate is calculated by the equation reported in Materials and Methods section.

A)



B)

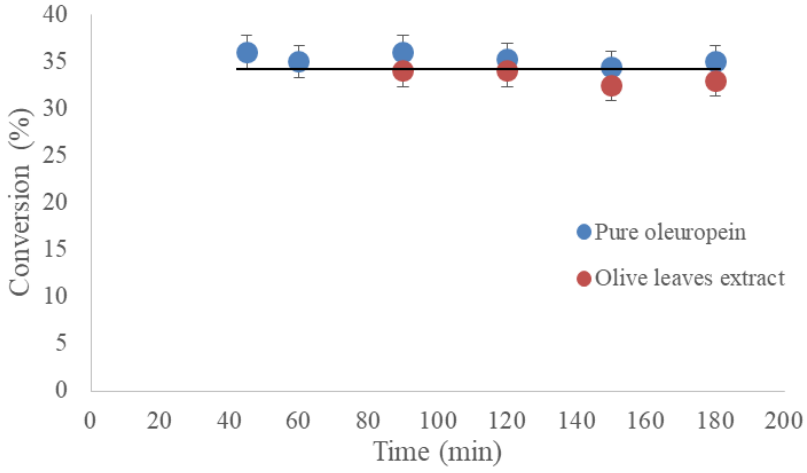


Fig. 4. Oleuropein concentration in a monophasic biocatalytic membrane reactor (a), and conversion obtained when the system was fed with pure oleuropein or with leaves extract containing oleuropein (b). Enzyme immobilized used: 5 mg.

A conversion of about 36 (± 4) % was obtained at each passage of oleuropein through the biocatalytic membrane (residence time: 0.6 min) with a reaction rate of 8.6×10^{-4} ($\pm1.0\times10^{-4}$) mmol/cm^{3}min.

The biocatalytic membrane was re-used for 6 reaction cycles and for a total observation period of about 18 hours, demonstrating constant performance as a function of time.

The same constant conversion was achieved by using olive leaves extract as the feed (Fig. 4b), confirming that the enzyme activity is not altered from the presence of other polyphenols and more importantly that the leaves extract can be used as source to produce phytotherapic compounds by a biocatalytic membrane system. Also by using this feed the reaction was repeated for 6 reaction cycles for a total observation period of 18 hours, and also in this case a constant conversion was obtained with no decay in system performance.

In the STR a conversion of about 50 % after 300 minutes was obtained, while the reaction rate is 1.8^{-4} mmol/cm^{3}min ($\pm$ 1.0^{-5}). In in this system enzyme inhibition by the other co-product (glucose) is present as demonstrated in supporting information. For this reason, in this reactor there is the problem to separate reagents when the maximum conversion was reached, which needs additional downstream processes. In this particular case the continuous removal of the product in the BMR is an additional advantage.

A comparison of the obtained results with another continuous biocatalytic membrane reactor [30], in which the enzyme was physically immobilized onto polymeric membranes, underlines that the covalent immobilization does not alter the enzyme catalytic activity since the enzyme has very similar reaction rate (physical entrapment: 6.9^{-4} ($\pm3^{-5}$) mmol/min·cm^3; present work: 6.8^{-4} ($\pm4^{-5}$) mmol/min·cm^3), in the case in which similar enzyme amount was immobilized. But it must be pointed out that about the same reaction rate value was obtained with a lower biocatalyst

concentration (physical entrapment 7.2^{-5} ($\pm 3^{-6}$) mmol/cm^3, present work: 5.5^{-5}($\pm 2^{-6}$) mmol/cm^3), indicating a good performance of the enzyme also when covalently immobilized onto a membrane. Besides, the covalent immobilization could preserve the catalytic system from the enzyme leaching during long-term operation.

3.3.1 Selection of organic solvent for multiphase biocatalytic membrane reactor

In Table 1 some properties of the different organic solvents tested for oleuropein extraction are summarized, together with their extraction capacity towards the compound of interest.

Results evidenced that oleuropein aglycone extraction occurs only in ethyl acetate and octanol. Indeed, about 47% of extraction was obtained in the case of octanol, while about 62% into ethyl acetate. An ideal organic solvent should be very slightly soluble in water and should have low boiling temperature and low toxicity. The low boiling temperature will promote an easy solvent evaporation, after separation from water phase, in permeate samples.

Table 1. Organic solvent properties and related extraction percentage of oleuropein aglycone from water

Organic solvent	**Solubility in water (%) v/v**	**Oleuropein aglycone extracted (%)***	**Boiling point (°C)**
Cyclohexane	0.01	0	81.0
Octanol	0.03	47	188.0
Ethyl Acetate	8	62	77.1
Squalene	Not soluble	0	275.0
Toluene	0.05	0	110.6
Dichloromethane	1.3	3	39.6

* 50:50 (v/v) organic/water phase (containing oleuropein aglycone) emulsion

Despite octanol has a lower solubility in water than ethyl acetate, its high boiling point (188°C) represents a problem for solvent removal and solid residue recovery. Thus, a lower boiling point, as in the case of ethyl acetate is preferred.

Besides, ethyl acetate is defined "recommended/preferred" solvent on the basis of green solvent choice criteria given by 'Registration, Evaluation, Authorisation and Restriction of Chemicals' (REACH) and the recommendations of pharmaceutical industry [31]. This will permit its direct use, after enriching it of polyphenols instead of other solvents, in food formulation production [32]. On the basis of the partition coefficient reported on Fig. 5, the ratio of the ethyl acetate/water to be used in the multiphase system to obtain a high extraction of the compound of interest was fixed to 1:5 (v/v).

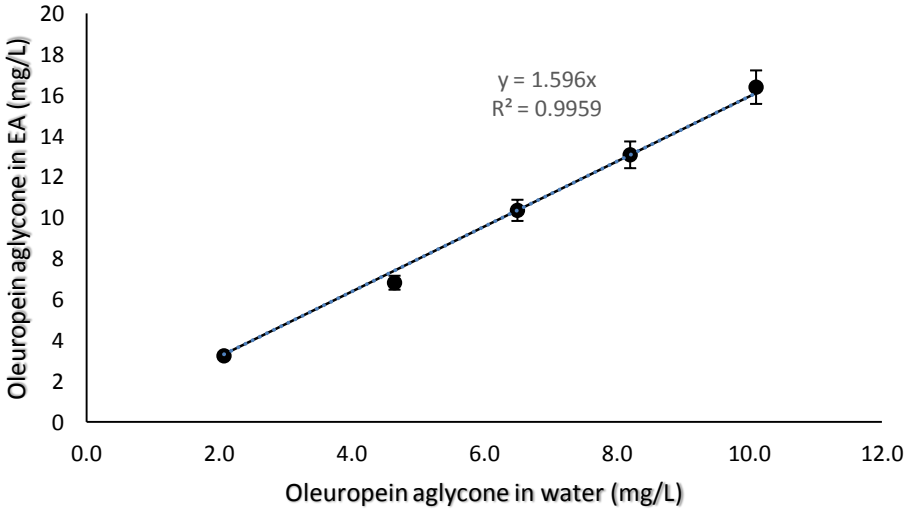


Fig. 5. Partition coefficient of oleuropein aglycone in ethyl acetate

The oleuropein hydrolysis in ceramic multiphase biocatalytic membrane reactor was carried out by using a ceramic membrane containing 5 mg of covalently immobilized β-glucosidase. The aqueous phase containing the oleuropein was recirculated into the shell of the system with an axial velocity of $4.2 \cdot 10^{-3}$ m/sec. It passed through the biocatalytic membrane at a flow rate of 0.3 ml/min, resulting in a residence time of about 0.96 min. On the other side of the membrane the ethyl acetate solution was recirculated with an axial velocity of 1.6×10^{-3} m/sec. So the aqueous solution containing the products met the organic phase in lumen and forms the unstable water-in-oil emulsion. In this case the ratio of water that passes through the membrane and ethyl acetate recovered into the permeate was 1/5 (v/v).

In order to evaluate the efficiency of the multiphasic biocatalytic membrane reactor, prior to recirculate the organic phase into the system, the performance of the immobilized β-glucosidase was studied under a single-phase condition. Once the steady-state was reached, the ethyl acetate was recirculated into the lumen side in order to promote the extraction of the oleuropein aglycone.

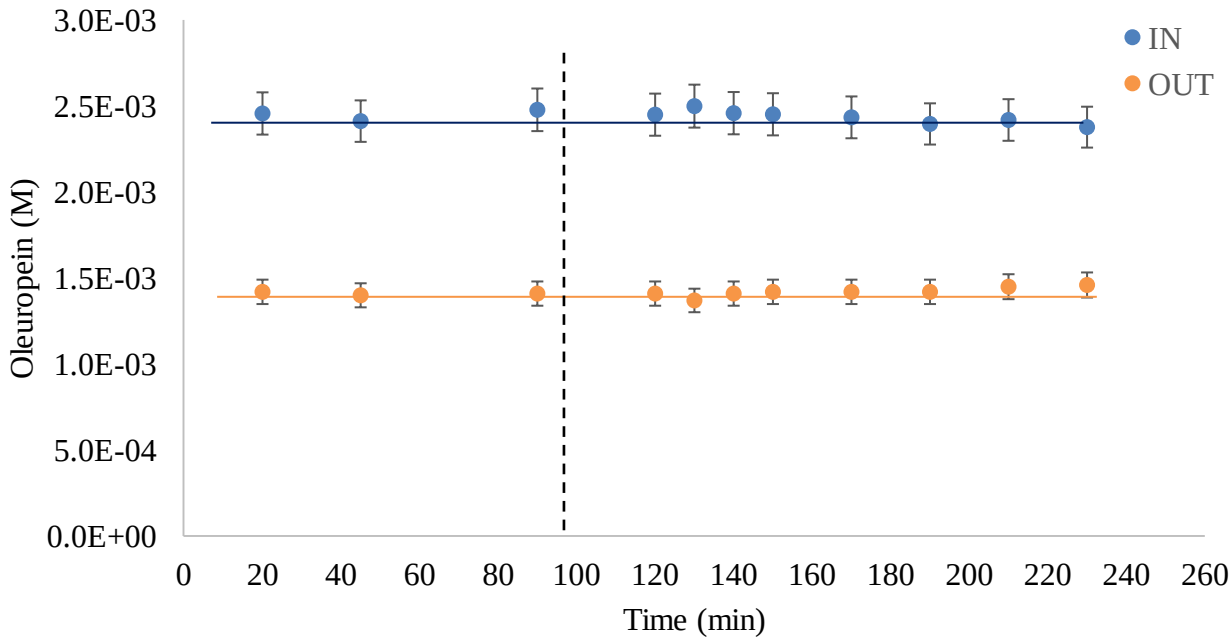


Fig. 6. Concentration profile of oleuropein that enters in the biocatalytic membrane (IN) and collected in the permeate phase after hydrolysis (OUT) in monophasic biocatalytic membrane reactor and in multiphasic biocatalytic membrane reactor. The black dashed line represents the time where it was started the recirculation of the organic phase.

Constant concentration profiles (Fig. 6) of oleuropein that enters into the biocatalytic membrane (IN) and the one collected into the aqueous phase in the permeate is obtained, confirming that the system works as a continuous system also when the organic phase was recirculated into the lumen side. Also in this case at each passage through the membrane a conversion of 36 (±2) % was reached. The ethyl acetate collected fractions were separated from the aqueous phase and evaporated, then the solid powder was suspended in buffer phase (50 mM phosphate buffer, pH 6.5) and the amount of oleuropein aglycone and oleuropein were measured again by HPLC. When the

oleuropein conversion was about 36 (±2) %, the extraction of the compound of interest into the organic solvent was 90% at each passage through the membrane, in accordance with the oleuropein aglycone partition coefficient (Fig.6). This high value of extraction percentage allows preserving the hydrophobic compound from a possible rearrangement in water.

Considering that the prize of polyphenols contained in olive oil, such as oleuropein aglycone, is very high (e.g. hydroxytyrosol about 2700 euro per gram, Extrasynthese), the development of a method to isolate in one step a non-commercially available compound with 90 % of extraction in a pure solvent could be highly advantageous from an economic point of view.

The further solvent removal permitted to obtain a solid powder enriched of oleuropein aglycone which has a major versatility respect to the dissolved forms and it could be used in several formulations in many fields. Besides, the solid form promotes higher aglycone stability, since powders are generally less reactive in comparison with solutions.

The evaporation process was used in this system to demonstrate the concept, but less energy intensive processes can be applied, such as solvent resistant nanofiltration coupled with membrane crystallization.

Conclusions

A multiphase biocatalytic membrane system was developed and compared with a monophasic membrane system for oleuropein aglycone production starting from pure oleuropein or olive leaves extract. The multiphase system permitted to produce and simultaneously extract the hydrophobic aglycone in a pure solvent. A solid powder enriched of oleuropein aglycone can be obtained by a subsequent solvent removal.

The intensified biocatalytic/extractor reactor proposed well respond to the need to develop “green systems”, since it promotes both the valorization of renewable material (olive leaves), and the production/stabilization of a high added value phytotherapic compound by a continuous process.

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Highlights

- Hydrophobic oleuropein aglycone is an antioxidant not commercially available

-It can be continuously produced from olive leaves by an intensified membrane process

-Multiphase biocatalytic membrane reactor allowed produce/extract the aglycone

-An unstable water-in-oil emulsion produced in the permeate allowed the extraction

- Enriched powder of oleuropein aglycone can be produced after solvent evaporation

Graphical abstract