

## Enzymes immobilized in advanced membrane reactor/separator for the production and stabilization of reaction intermediates

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In this work, the catalytic performance of a biocatalytic membrane reactor using  $\beta$ -glucosidase immobilized on polysulfone capillary membranes was investigated. The membrane reactor was used for the continuous hydrolysis of oleuropein (an abundant vegetable raw material) into aglycon (a powerful natural antimicrobial agent) and glucose. In addition, the membrane reactor system simultaneously performed their separation from the reaction microenvironment. The study of the kinetic properties of the heterogenized enzyme showed that enzyme activity and stability can be preserved and that the reverse relationship observed in the literature is not a general rule. In fact the kinetic parameters of the immobilized enzyme were the same as the ones of the free enzyme.

The distribution of the enzyme inside the polymeric hollow fibers membranes and its catalytic activity were evaluated by an *in situ* method applying a combined technique merged from molecular biology techniques, such as colorimetric enzyme activity and western blotting.

Biocatalytic membranes with heterogenized  $\beta$ -glucosidase from almond were then used to develop an advanced biocatalytic membrane reactor working both in aqueous phase and or in multiphasic aqueous/organic system. The aim was to hydrolyze oleuropein to produce phytochemicals not commercially available such as the isomer of oleuropein aglycon. The isomer of oleuropein aglycon is produced in the first step of oleuropein hydrolysis but is present only in the olive oil due to its low stability in water. Even is the strong antioxidant properties of this compound are very well known in olive oil, the substance was not yet purified and it's not commercially available. Results obtained showed that in the multiphasic aqueous/organic system there is the production of the isomer of oleuropein aglycon and its simultaneous isolation into the organic phase.

## 2. Introduction

Biocatalytic membrane reactors are systems in which it is possible to integrate and intensify biochemical transformation with transport phenomena in single system. The biochemical transformation is carried out thanks to a biological system while the transport is governed by a membrane. The process is governed by controlled fluid dynamic conditions. Despite the various fields of applications, such as pharmaceutical,

food and biotechnology the application at industrial scale is still limited and more appropriate research effort in this field is needed.

The versatility of the technology offers the possibility to integrate and intensify the production process due to the presence of different compartments: external, membrane, internal. The possibility to have different compartments suggests the potential use of different phases with different properties, that are simultaneously separated but in contact.

This is the system needed for example in the case in which the hydrolysis reaction products of interest are not stable in water, so for the production and the simultaneous extraction a multiphasic biological system is needed.

The isomer of oleuropein aglycon (3,4-DHPEA-EA) is a molecule not stable in water (Rodis et al 2002), and together with hydroxytyrosol, represents the compound responsible of the antioxidant properties of olive oil. Different works present in literature (Guiso and Marra, 2005) demonstrated that 3,4-DHPEA-EA is produced during the oleuropein hydrolysis in olives, by the action of  $\beta$ -glucosidase. Oleuropein and  $\beta$ -glucosidase are compartmentalized in different places inside the olives (Morant et al. 2008) and the mechanical force applied during crushing permits the contact between them and consequently also the enzymatic reaction.

The oleuropein aglycon is produced during the first step of the oleuropein hydrolysis, but due to its low water solubility it is fastly partitioned in the oil phase, this is the reason why it is founded only in olive oil and not in vegetation water. If no oil phase is present the aglycon molecule is fastly rearranged into molecules stable in water. The possibility to produce and isolate the isomer of oleuropein aglycon it is of high interest in view of its potential application due to its high antioxidant properties and also as standard to characterized different olive oil.

In this work the possibility to hydrolyze oleuropein by the action of heterogenized commercial  $\beta$ -glucosidase was evaluated. The system was then implemented coupling the  $\beta$ -glucosidase membrane reactor with an extractive multiphasic system to isolate the 3,4-DHPEA-EA in the organic phase.

### 3. Materials and methods

The main steps involved in the development of the biocatalytic membrane reactor to produce and isolate the compound not stable in water are: i)  $\beta$ -glucosidase immobilization by physical entrapment ii) characterization of the biocatalytic membranes by measurement of the activity *in situ* of the heterogenized enzyme and by immunolocalization iii) measurement of the kinetic parameters in a biocatalytic membrane reactor., iiiii) development of a continuous stirred membrane reactor working both in aqueous and in aqueous/organic system for the production and the simultaneous extraction of the compound of interest not stable in water.

#### 3.1 Chemicals and methods

$\beta$ -glucosidase from almond and glucose were obtained from Sigma Aldrich, oleuropein from Extrasynthese (France). Acetonitrile and *o*-phosphoric acid for HPLC mobile phase preparation were purchased from Carlo Erba and Sigma Aldrich, respectively.

The methods used to characterize the reaction products were HPLC. The column used is a reverse silica LiChoCART Superspher RP8 column, 250-4 mm, 5  $\mu$ m (Merck). The

mobile phase was a mixture of acetonitrile/water (21:79) acidified with *o*-phosphoric acid (up to pH 3). The flow rate and pressure were 0.8 ml/min and 210 ( $\pm$  4) bar, respectively. The oleuropein was detected at 280 nm wavelength. The measurement of glucose was measured using a reagent kit (glucose HK assay kit, Sigma Aldrich) by means of a spectrophotometric method based on the absorbance of NADH at 340 nm. The measurement of the compound not stable (isomer of oleuropein aglycon) in water was measured indirectly by monitoring the disappearance of the oleuropein aglycon rearrangements reaction products in water, combining the measurement by HPLC and the results obtained by TLC. The TLC method was conducted using the method reported in Briante et al. (2000). Analysing oleuropein concentration by HPLC in the reaction samples, it is possible to detect the peak that corresponds to the oleuropein aglycon rearrangements product in water.

The membrane used were polysulphone hollow fiber asymmetric membrane of 30 kDa nominal molecular weight cut-off (NMWCO). The membrane void volume, that corresponds to the reactor volume is 0.61 ( $\pm$  0.02) cm<sup>3</sup>.

### 3.2 Equipments

To measure the kinetic parameters of the biocatalytic membrane the system used is illustrated in Fig.1a. A peristaltic pump was used to feed the solution to the biocatalytic membrane, the inlet/outlet pressure was measured by pressure gauges (PG). The system used to conduct the oleuropein hydrolysis and to simultaneous extract the isomer of oleuropein aglycon was illustrated in Fig. 1b. The organic phase was recirculated by a peristaltic pump in the lumen side of the membrane with a flow rate of 0.24 ml/min. The oleuropein aqueous phase passes through the membrane, where the enzyme is present so it was converted into the reaction products: glucose and oleuropein aglycon. The aqueous phase containing the reaction products passing through the membrane meets the organic phase present into the lumen side, in this way the different compound are distributed into the two phases basing on its solubility. The two phases are immiscible so they are recovered into the permeate separately and ready for the analysis.

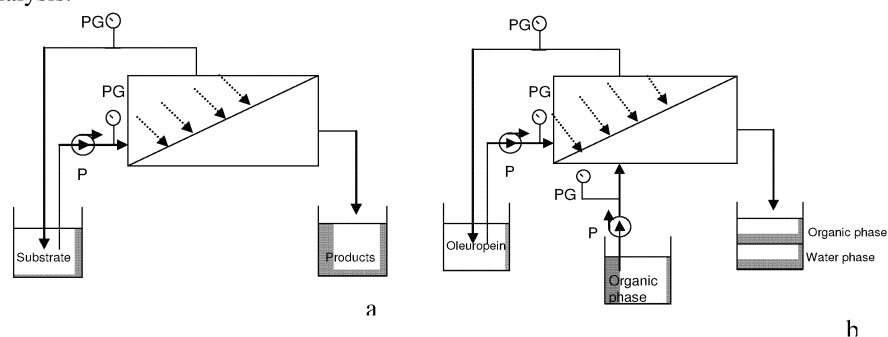


Fig.1 Schematic representation of equipments. Continuous membrane reactor working in homogeneous aqueous system (a), and in multiphasic aqueous/organic system (b). PG: pressure gauges, P: peristaltic pump

### 3.3 Immobilization procedure and characterization

The initial concentration of the enzyme was 0.035 mg/ml. The enzyme was immobilized on the membrane applying a transmembrane pressure of 0.35 bar. When

the enzyme was fed to the asymmetric membrane (30 kDa MWCO) due to its molecular weight (65 kDa) it cannot pass through and remains entrapped. The immobilization procedure was stopped when the flux, measured during the time, reached the steady-state. The absence of the protein passage through the membrane was confirmed carrying out SDS-page on the collected fractions.

The spatial distribution of the enzyme on the membrane was evaluated by using a synthetic substrate that precipitates where the enzyme is active showing by blue color the activity *in situ*. The activity *in situ* was combined with a spatial localization using polyclonal antibody. By the use of this combined method fully explained in Mazzuca et al. (2006) it was possible to see where the enzyme is located and where it is active directly on the membrane by optical microscopy. The biocatalytic membrane were then used to develop a biocatalytic membrane reactor to measure the kinetic parameters towards oleuropein.

### 3.1 Kinetic parameters measurement in biocatalytic membrane reactor and comparison with a stirred tank bioreactor

Kinetic parameters were measured using the following oleuropein concentrations : 1, 2, 2.5, 5, 7.5, 10 and 15 mM at 25°C and pH 6.5. The results obtained were compared with a stirred tank bioreactor, using the same reaction conditions as in biocatalytic membrane reactor.

### 3.2 Development of a biocatalytic membrane system working both in aqueous and in multiphase aqueous/organic system

The  $\beta$ -glucosidase membrane reactor tested for the hydrolysis of oleuropein and characterized in terms of kinetic measurements was then used to implement the system developing a combined biocatalytic membrane reactor working in multiphase system.

## 4. Results and discussions

### 4.1 Immobilization procedure and characterization

During the enzyme immobilization the flux reached the steady-state at about 50 l/hm<sup>2</sup>, after which the immobilization process is stopped.

The characterization of biocatalytic membranes by the combined method, measurement of the activity *in situ* and the determination of enzyme spatial distribution, reported in Mazzuca et al. (2006) showed that (dark spots) there is a random distribution and orientation of the protein within the membrane (Fig. 2). The catalytic activity, using oleuropein as substrate did not show any significant decay for about 30 h operation time. The enzyme remained still active also measuring the activity after two months, with an intermediate refrigeration at -80°C.

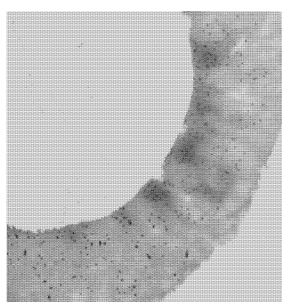


Fig. 2 Cross-section of  $\beta$ -glucosidase from almond loaded-membrane, after combined methods of immunolocalization and *in situ* activity assay observed by optical microscopy

#### 4.2 Kinetic parameters measurement in biocatalytic membrane reactor and comparison with a stirred tank bioreactor

$K_m$  was measured in the biocatalytic membrane reactor and in a stirred tank bioreactor. The results obtained showed that the immobilization technique don't alter the catalytic properties of the heterogenized enzyme.  $K_m$  is of the same order of magnitude in the two bioreactors (Fig. 3) demonstrating that the lower catalytic activity observed for immobilized enzyme is not a general rule.

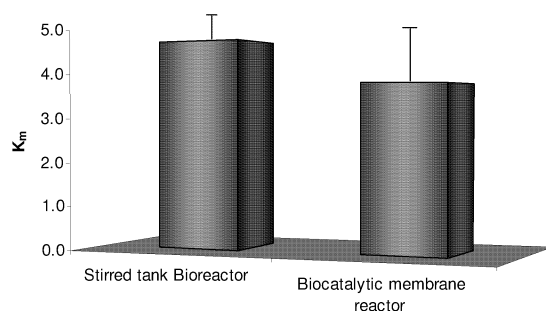


Fig. 3  $K_m$  (mM) of stirred tank reactor and biocatalytic membrane reactor with heterogenized  $\beta$ -glucosidase.

#### 4.3 Development of a biocatalytic membrane reactor working either in aqueous and in multiphasic aqueous/organic system

The  $\beta$ -glucosidase membrane reactor characterized in terms of kinetic parameters measurement was then used to develop system that worked both on aqueous or in multiphase system. The organic phase used and recirculated into the lumen side of the biocatalytic membranes was limonene. To monitor the production and the simultaneous extraction of isomer of oleuropein aglycon, not stable in water, the aqueous phase was analyzed by HPLC, in which together with oleuropein is it possible to detect the rearrangement products in water phase of oleuropein aglycon. In the case in which the

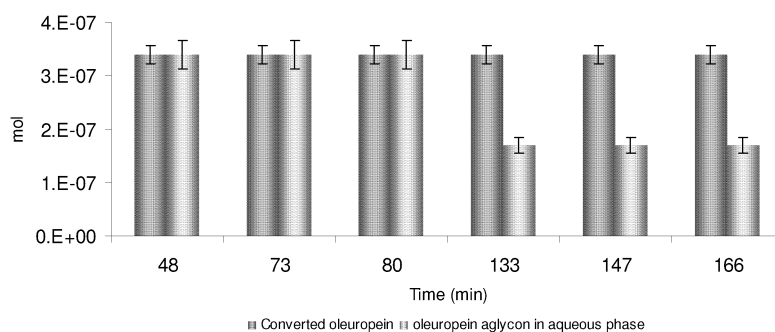


Fig. 4 Comparison between moles of converted oleuropein and oleuropein aglycon in aqueous phase produced in biocatalytic membrane reactor in homogeneous aqueous phase (BMR-Aqueous phase) and in biocatalytic membrane reactor in multiphasic aqueous organic system.

biocatalytic membrane reactor works only in the aqueous phase the moles of converted oleuropein and the moles of produced rearrangements products soluble in water are the same as reported in the Fig. 4. In the case in which the biocatalytic membrane reactor worked in multiphasic system, the moles of oleuropein and the moles of oleuropein aglycon rearrangement products are not the same. This decrease (Fig 4) of the product is due to the fact that oleuropein aglycon produced in the first step it is simultaneously entrapped into the organic phase. In both systems the moles of converted oleuropein and the moles of glucose, produced together oleuropein aglycon in the first reaction step, are the same.

## 5. Conclusions

$\beta$ -glucosidase-loaded membranes were used to build-up continuous membrane bioreactors working both in aqueous and in multiphasic system. This system is used to hydrolyze oleuropein, compound also present in waste material of olive oil waste and in renewable material such as olive leaves. The aim was to produce and isolate water unstable reaction product such as oleuropein aglycon, that represents one of the most important antioxidant compound present in olive oil, not yet purified and not commercially available.

Results obtained evidenced the possibility that the biocatalytic membrane reactor working in multiphasic system can isolate, after the production, the isomer of oleuropein aglycon in the organic phase.

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