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Ph.D. Thesis

Study and development of biocatalytic membrane reactors as biomimic plant cell systems for the production and separation of bioactive molecules from renewable materials

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Contents

Summary……………………………………………………………………..5
Sommario………………………………………………………………………11

Biological system…………………………………………………………………19

Chapter 1: Biophenols in virgin olive oil and compartimentalization as defence mechanism in plant as model to develop alternative biotechnological processes…21

Bioartificial membrane systems biomimiking *in vivo* processes……………43

Chapter 2: Clustering of interactions between bioactive macromolecules and membranes, and identification of resulting properties of the nanostructured biohybrid membrane systems…………………………………………………… 45

Chapter 3: Principles of Membrane Bioreactors and Membrane emulsification process………………………………………………..……..63

Chapter 4: Biochemical Membrane reactors in industrial process and in patent development…………………………………………………………………..79

Chapter 5: Improvement of β-glucosidase activity of *Olea europaea* fruit extracts processed by membrane technology………………………………105

Chapter 6: Immunolocalization of β-glucosidase immobilized within polysulphone capillary membrane and evaluation of its activity *in situ*…………121

Chapter 7: Kinetic study of a biocatalytic membrane reactor containing immobilized β-glucosidase for the hydrolysis of oleuropein…………………….137

Chapter 8: Biocatalytic membrane reactor and membrane emulsification concepts combined in a single unit to assist production and separation of water unstable reaction products…………………………………………………159

Conclusions……………………………………………………………173
Appendix………………………………………………………………175

Summary

Living cells are divided into several compartments by a membrane unit. Each compartment has a specific structure and function and the interactions between compartments are regulated by specific signals. One important example of compartimentalization regulated by *stimuli*-responsive action is the defence plant against pathogens.

Through the course of evolution, plants have become nature's organic chemists par excellence, and collectively synthesize a plethora of secondary metabolites to defend themselves against herbivores and microorganisms and adapt to different types of abiotic environmental stresses.

Some plant secondary metabolites are classified as phytoanticipins. When plant tissue in which they are present is disrupted, the phytoanticipins are bio-activated by the action of β-glucosidases. These binary compartments – two sets of components that when separated are relatively inert– provide plants with an immediate chemical defense against protruding herbivores and pathogens.

β-glucosidases action is also involved in phenolic compounds production during olive oil processing in the step of olives crushing. The mechanical destruction of the two compartments containing enzyme/glucosides causes the hydrolysis of glucosides present in olives and the partition of phenolic compounds produced in olive oil or aqueous phase basing on its partition coefficient. The phenolics compounds produced in this hydrolysis are very important for their high antioxidant properties and in view of their potentially use in pharmaceutical and food industry.

The main idea of the work presented was to simulate the different compartments present in vegetal system, in which β-glucosidase action is involved, by creating a bioartificial system to able produce high added value compounds under controlled conditions. Due their potential application in the production of antioxidant compounds present in olive oil, the enzyme/substrate studied is the biological system β-glucosidase/oleuropein.

The biotechnology used to conduct the hydrolysis of oleuropein is a biocatalytic membrane reactor.

Nanostructured microporous membranes functionalized with biomolecules are very suitable for the development of biohybrid and biomimetic systems which can be used in various field including biocatalysis, biomedicine and biotechnology. The system simulates the biological membrane environmental like, where the enzyme is heterogenized inside the membrane and the passage of the substrate is regulated by controlled fluid dynamic conditions.

In addition this technology permitted to work in mild conditions, the equipments need small space, are flexible and easy to scale-up (they are enabling technologies and well respond to the process intensification strategy); products are of high quality; co-products are also of high quality.

The development of biohybrid system for the hydrolysis of oleuropein is of high interest also because this compound is contained in waste material coming from olive oil processing and in renewable material such as leaves.

Nowadays, plant materials represent the major renewable resource for human food production and consumption. In particular, vegetable oils production covers a huge sector of food market. The annual production of vegetable oil is more than one million tons in European Union (FAOstat database, 2006 and 2007). In all industrial production sectors, significant amount of wastes and by-products containing high added value bioactive molecules are produced. If discharged, these wastes represent a serious contamination source of environmental because of their BOD and COD content. On the other hand if properly processed and recovered, they represent a sustainable renewable source of high added value compounds. These unique property compounds, in some cases are economically more valuable than the product from whose processing line they come from.

In fact, wastes and by-product contain a huge amount of high added value bioactive components as valorisable substances from which novel products with key nutritional, therapeutic, defence properties can be obtained.

The recovery and valorisation of these compounds is of high interest for high quality of stable food formulation as well as for sustainable industrial production approach to zero discharge. This will also respond to the challenges of scarse or limited renewable resources, need for new property food in modern diet requirement, environment protection through waste prevention and minimization.

The work carried out includes the study of biological systems and biomolecules from vegetal plant as well as the development of bioartificial systems able to use and mimic biological systems. The developed biohybrid systems have been tested for the production of high valuable biomolecules starting from side-products obtained in olive oil production. The various aspects of the work are summarized in the following while the main strategy is reported in the flowsheet Fig.1; furthermore for each of the indicated items an extensive description is reported in the various chapters as summarized in the following:

Biological system:

- Biophenols in virgin olive oil and compartimentalization as defence mechanism in plant as model to develop alternative biotechnological processes.

The compartimentalization present in plant material is involved in plant defence against pathogens and in the production of biophenols present in olive oil, responsible for its high antioxidant properties. In both processes the action of β-glucosidase on phenolic compounds is necessary. In this chapter 1 the importance of phenols present in olive oil produced with the same enzymatic mechanism, their antioxidant properties and their biogenesis are highlighted. The different compartimentalization involved in plant defence against pathogens in which β-glucosidase and glucosides are involved is also described in view of a possible simulation model to develop the biomimic process by membrane technology. The recovery of these compounds starting from waste source or renewable material is also very important for a sustainable industrial production. For this reason also the possible raw source material from which they can be extracted is reported.

Bioartificial membrane system:

- The experimental work in biocatalytic membrane development was supported from literature analysis about membrane and biomolecules interactions reported in chapter 2. In particular the study highlights the properties (biochemical, biophysical) and techniques to characterize 1) bio-macromolecules (i.e. proteins) and membranes; 2) bio-macromolecules-membrane interactions; and 3) resulting biohybrid systems.

-Biomimiking hybrid bioartificial membrane system

Basing on the study carried out about the biological system and the interaction between membrane and biomolecule, the second part of the work was the development of an artificial system that recreated the compartments normally present *in vivo*. The different compartments present in biocatalytic membrane reactor working under permeating conditions, permitted to conduct the enzymatic hydrolysis using a heterogenized biocatalytst and to control the enzymatic reaction under controlled fluid-dynamic conditions. To apply this technology in the field of interest the principles and the different configuration used in membrane bioreactor were analyzed in chapter 3. In addition, in order to evaluate the possibility to exploit biohybrid membrane systems with catalytic properties, the analysis of development level in different applications have been carried out and reported in terms of industrial application and patent development chapter 4.

- Development of biocatalytic membranes

To develop biocatalytic membranes for the hydrolysis of oleuropein two different enzymes were heterogenized on membranes: β-glucosidase from almond, the commercial enzyme, and the natural enzyme β-glucosidase extracted from olive fruit.

Due to the fact that β-glucosidase from olive fruit is not commercial available but obtainable only as complex extract the purification of the natural enzyme extract from olive fruit was necessary and was carried out applying membrane technology. The part concerning this work is described in chapter 5.

The commercial enzyme from almond and the purified enzyme extracted from olive fruit were then immobilized on polymeric membrane and the catalytic efficiency of the heterogenized system was evaluated measuring the activity *in situ* chapter 6, while the spatial distribution of the enzyme on the membrane was carried out by immunolocalization.

- Characterization of biocatalytic membrane reactor for the hydrolysis of oleuropein

The development of the biocatalytic membrane reactor for the hydrolysis of oleuropein was then carried out by the use of the commercial enzyme from almond. The catalytic property of the heterogenized system was evaluated in terms of kinetic parameters measurements. The biocatalytic membrane reactor for the oleuropein hydrolysis was also compared with a batch bioreactor. The detailed work was reported in chapter 7

- Bioartificial Membrane system that biomimiking the *in vivo* process

The biocatalytic membrane reactors developed and tested for the hydrolysis of oleuropein was then used to conduct the hydrolysis of oleuropein and to simultaneous produce and isolate the antioxidant phenolic compound of interest not stable in water and present in olive oil. The system simulates not only the different compartimentalization present in *in vivo* system, but also the different phases that are present in olives during olive oil production: oil and aqueous phase. In this way the important antioxidant molecule present in olive oil can be produced in aqueous phase and isolated from the other reaction products into the organic phase.

The possibility to design and proof the concept of an integrated and intensified membrane system able to carry out in a single operation unit, bioconversion in water phase, and simultaneous separation of reaction products having different solubility and stability in water, was carried out by creating water in oil emulsion. To achieve this aim, the concepts of biocatalytic membrane reactor and membrane emulsification have been integrated so that to

build-up a combined membrane operation system where hydrolysis occurred within the microporous membrane structure (that contained immobilized enzyme) and extraction occurred at the membrane interface where the permeating water reaction phase was collected as droplets into the organic phase circulated along the lumen membrane surface (chapter 8).

Sommario

Le cellule viventi sono sistemi compartimentalizzati altamente funzionali. Ogni compartimento è delimitato da una membrana ed è caratterizzato da una specifica struttura e funzione. La comunicazione tra l'esterno e l'interno del compartimento e tra i diversi compartimenti è regolata da specifici segnali in risposta ad uno stimolo.

Un importante esempio di regolazione compartimentalizzata regolata da stimolo è il sistema di difesa delle piante nei confronti dei patogeni.

Nel corso dell'evoluzione, le piante, per sopravvivere agli stresses di tipo biotico ed abiotico, sono diventate "chimici organici" naturali per eccellenza, attraverso la sintesi di un'ampia serie di metaboliti secondari.

Alcuni di questi metaboliti secondari compartimentalizzati all'interno della cellula vegetale sono le fitoanticipine. Questi composti spesso si trovano in forma glucosilata e vengono bioattivati dall'azione idrolitica della β-glucosidasi, enzima a sua volta compartimentalizzato all'interno della cellula.

Quando il tessuto della pianta subisce una rottura meccanica, a causa di un patogeno (es. una puntura di insetto), i compartimenti vengono distrutti e le fitoanticipine sono bioattivate dalle β-glucosidasi. Il sistema binario, compartimenti enzima/compatimento substarto, è un sistema che se non in contatto è relativamente inerte, ma quando attivato fornisce alla pianta una difesa chimica immediata contro l'attacco patogeno.

L'enzima β-glucosidasi è anche coinvolto nella produzione dei composti fenolici dell'olio d'oliva durante il processo di spremitura delle olive.

La spremitura delle olive è un altro sistema di stimolo meccanico che provoca la distruzione dei due compartimenti enzima/biofenolo, con successiva idrolisi del composto e conseguente ripartizione dei prodotti tra la fase olio e l'acqua (acqua di vegetazione). I composti fenolici prodotti durante questa reazione di idrolisi sono molto importanti in quanto responsabili del potere antiossidante dell'olio d'oliva.

L'obiettivo principale del seguente lavoro è stato quello di simulare i diversi compartimenti presenti nella cellula vegetale, in cui è coinvolta l'azione della β-glucosidasi sul biofenolo oleuropeina, attraverso la creazione di un sistema artificiale a membrana biocompatibile. Lo scopo finale è quello di produrre e isolare composti antiossidanti contenuti nell'olio d'oliva, ma non ancora isolati per un futuro utilizzo nell'industria farmaceutica ed alimentare.

La biotecnologia utilizzata per condurre l'idrolisi dell'oleuropeina è un sistema biocatalitico a membrana.

Membrane artificiali microporose e nanostrutturate funzionalizzate con biomolecole sono particolarmente adatte allo sviluppo di sistemi bioibridi e biomimetici potenzialmente utilizzabili in diversi settori tra cui biocatalisi, biomedicina e biotecnologia. Il sistema artificiale a membrana simula l'ambiente naturale delle membrane biologiche, l'enzima è eterogeneizzato alla membrana e il passaggio del substrato è regolato da condizioni fluido dinamiche estremamente controllate. I sistemi biocatalitici a membrana permettono inoltre di lavorare in condizioni blande di temperatura e pH, gli impianti necessitano di poco spazio sono flessibili e facili da sviluppare in processi di "scale-up", con prodotti e co-prodotti di alta qualità.

Lo sviluppo di sistemi bioibridi per l'idrolisi dell'oleuropeina è importante anche per un potenziale sviluppo a livello industriale nella logica dell'intensificazione di processo. Questo composto è contenuto in alta percentuale sia nel materiale di scarto prodotto durante la produzione dell'olio d'oliva (acque di vegetazione) che in materiale rinnovabile come le foglie dell'ulivo.

Oggi le piante sono considerate la maggiore risorsa rinnovabile per produrre sostanze destinate al consumo di alimenti e alla loro produzione.

In particolare la produzione di oli di natura vegetale copre un ampio settore del mercato alimentare, che equivale in Europa ad un milione di tonnellate all'anno (FAOstat database, 2006-2007).

In tutti i processi industriali sono prodotte significative quantità di scarti e co-prodotti contenenti molecole bioattive ad alto valore aggiunto. Se semplicemente smaltiti nell'ambiente rappresentano una grave fonte di contaminazione a causa dell'alto contenuto di BOD e COD. D'altra parte se processati in modo appropriato rappresentano un'importante fonte rinnovabile di composti di alto valore. Molto spesso i composti ottenuti hanno un maggiore valore economico dei prodotti da cui derivano.

Il recupero e la valorizzazione di questi composti è importante per la produzione di nuove formulazioni alimentari e anche per lo sviluppo di processi industriali sostenibili.

Questo processo allo stesso tempo risponde alla necessità di massimizzare l'utilizzo delle risorse rinnovabili, necessarie per la produzione di alimenti con proprietà aggiuntive e di salvaguardare l'ambiente attraverso la riduzione degli scarti.

Nel presente lavoro di tesi, le ricerche condotte hanno riguardato sia lo studio di sistemi biologici e biomolecole di origine vegetale sia lo sviluppo di sistemi bioartificiali in grado di impiegare e mimare i sistemi biologici. Tali sistemi bioibridi sono stati testati per la produzione di biomolecole ad alto valore aggiunto partendo da sottoprodotti ottenuti durante

la produzione di olio d'oliva. I vari aspetti del lavoro sono riportati di seguito, mentre la strategia complessiva del lavoro è riportata nello schema in Fig. 1. La descrizione delle varie unità in modo approfondito è riportata nei vari capitoli.

Sistema biologico

- Biofenoli presenti nell'olio d'oliva e sistema di compartimentalizzazione delle piante per la difesa contro i patogeni come sistema modello per lo sviluppo di processi biotecnologici alternativi.

La compartimentalizzazione presente nelle cellule vegetali, è un sistema su cui si basa sia la difesa delle piante dall'attacco patogeno sia la produzione di composti fenolici presenti dell'olio d'oliva. In entrambi i processi è coinvolta l'azione idrolitica della β-glucosidasi sui composti fenolici.

Nel capitolo 1 sono stati riportati l'importanza dei composti fenolici preseti nell'olio extravergine d'oliva, le proprietà antiossidanti e la loro biogenesi.

Inoltre la spiegazione della diversa compartimentalizzazione coinvolta nei sistemi di difesa contro i patogeni viene descritta come potenziale modello per lo sviluppo del sistema bioartificiale.

Nel capitolo 1 sono stati riportati anche alcuni esempi di prodotti di scarto (acque di vegetazione) e di materiali rinnovabili (foglie d'ulivo) contenenti composti fenolici, utilizzabili come fonte di recupero di oleuropeina (substrato della reazione), per evidenziare la possibilità di un futuro sviluppo di processo industriale sostenibile.

Sistema bioartificiale a membrana

- Il lavoro sperimentale per lo sviluppo del sistema bioartificiale è stato supportato dallo studio della letteratura inerente all'interazione delle membrane artificiali e le biomolecole (capitolo 2). In particolare lo studio riportato nel capitolo 2 evidenzia le proprietà (biochimiche e biofisiche) e le tecniche per caratterizzare i) bio-macromolecole (proteine) e membrane, ii) le interazioni bio-macromolecole-membrane, iii) e i sistemi bioibridi.

- La seconda parte del lavoro ha riguardato lo sviluppo del sistema bioartificiale che ricrea i compartimenti presenti nel sistema *in vivo*. I diversi compartimenti presenti nei reattori biocatalitici a membrana, permettono una spaziale separazione tra il substrato della reazione, l'enzima eterogeneizzato sul supporto polimerico, e i prodotti. L'idrolisi inoltre è condotta in condizioni fluido dinamiche estremamente controllate.

Per poter applicare la tecnologia al settore di interesse sono stati analizzati e riportati nel capitolo 3 i principi e le principali configurazioni dei bioreattori a membrana. Nello stesso capitolo sono anche riportati alcuni concetti del processo di emulsificazione a membrana necessari alla comprensione del sistema integrato riportato nel capitolo 8.
Inoltre per valutare il grado di sviluppo della tecnologia a livello industriale, è stata condotta un'analisi nelle diverse applicazioni (capitolo 4), riportata come sviluppo di brevetti e sviluppo di applicazioni industriali.

Sviluppo di membrane biocatalitiche

Per lo sviluppo delle membrane biocatalitiche sono stati utilizzati due diversi tipi di enzimi: la β-glucosidasi estratta da frutto di olivo la β-glucosidasi commerciale di mandorlo.
β-glucosidasi estratta da frutto di olivo non è disponibile commercialmente, poiché i comuni metodi di estrazione modificano negativamente l'attività dell'enzima, di conseguenza è possibile ottenere l'enzima in un estratto proteico non purificato. Per poter sviluppare la membrana biocatalitica contenente la β-glucosidasi di olivo è stato necessario purificare l'enzima mediante tecnologia a membrana, il lavoro riguardante questa parte è riportato nel capitolo 5.
Entrambi gli enzimi di mandorlo e di ulivo sono stati utilizzati per la produzione di membrane biocatalitiche. L'efficienza del sistema eterogeneizzato a membrana è stata valutata mediante l'applicazione di una nuova tecnica in grado di misurare la localizzazione spaziale dell'enzima nella membrana e la misura della sua attività *in situ .)*
Il lavoro riguardante questa parte è riportato nel capitolo 6.

Caratterizzazione del reattore biocatalitico a membrana per l'idrolisi dell'oleuropeina.

Lo sviluppo del reattore biocatalitico a membrana per l'idrolisi dell'oleuropeina è stato condotto utilizzando l'enzima commerciale. Le proprietà catalitiche del sistema eterogeneizzato sono state valutate in termini di misura dei parametri cinetici. Il lavoro in dettaglio è riportato nel capitolo 7.

Sviluppo del sistema bioartificiale a membrana che simula l'ambiente *in vivo.*

Il reattore biocatalitico a membrana precedentemente caratterizzato è stato di seguito utilizzato per ricreare l'ambiente *in vivo*, sia per quanto riguarda la compartimentalizzazione che il sistema bifasico acqua/olio presente nelle olive. Lo scopo finale è quello di condurre l'idrolisi dell'oleuropeina in fase acquosa e simultaneamente isolare in fase organica composti

presenti nell'olio d'oliva non stabili in acqua, separandoli dagli altri prodotti di reazione solubili in acqua. Per ricreare questo sistema multicompartimento e multifasico sono state integrate due tecnologie a membrana il reattore biocatalitico a membrana e un processo di emulsificazione a membrana. L'integrazione del processo ha permesso di condurre l'idrolisi a livello della membrana biocatalitica dove è presente l'enzima eterogeneizzato. La fase acquosa proveniente dalla membrana biocatalitica e contenente i prodotti di reazione incontra istantaneamente la fase organica dove appunto avviene l'estrazione del composto di interesse. Il lavoro in dettaglio è riportato nel capitolo 8.

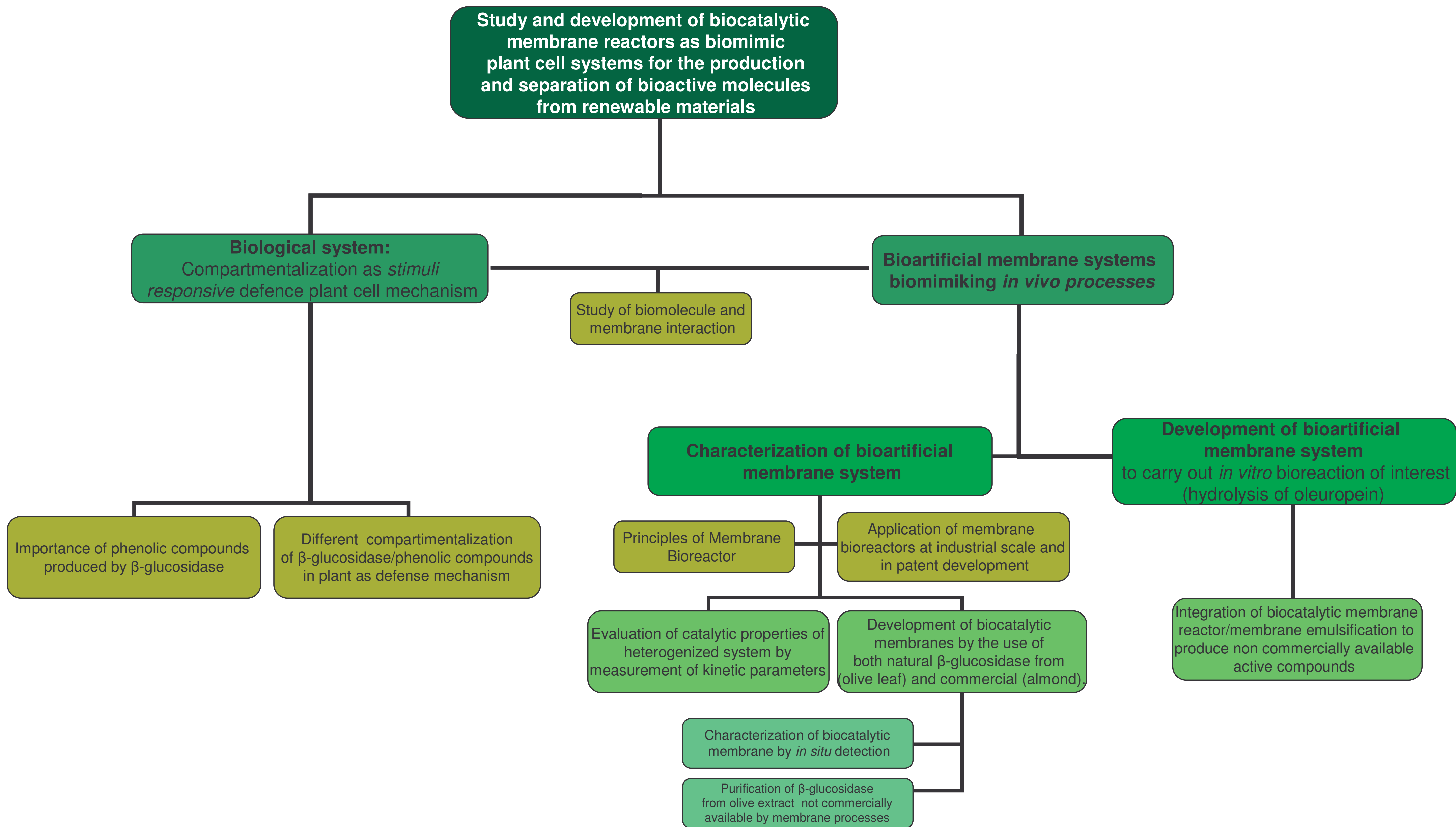


Fig. 1 Flowsheet illustrating the overall strategy of the work carried out

Biological system

Chapter 1

Biophenols in virgin olive oil and compartimentalization as defence mechanism in plant as model to develop alternative biotechnological processes

Abstract

Phenolic compounds are important substances present in olive oil. Their production is involved also in plant defence against pathogens. Both their production during olive oil processing that in plant defence against pathogens involve the hydrolytic enzyme β-glucosidase mechanism. In plant tissue β-glucosidase and its phenolic substrate are enclosed in different compartments activated by a mechanical (crushing of olive) or a biological (pathogen attack) *stimulus*. In this chapter the composition of phenolic compounds in olive oil, their health, antioxidant, organoleptic properties have been described.

One of the current problem for developing rapid and reproducible analysis of phenolic compounds is the absence of suitable pure standards. In view of the potential application of phenolic compounds in pharmaceutical and food field a deep understanding about the system *in vivo* is necessary and here reported together with the phenolic compounds biogenesis. In addition the renewable material from which phenolic compounds can be recovered it is reported.

1.1. Composition of phenolic compounds in Virgin Olive Oil

Polyphenols is a broad term used in the natural products literature to define substances that possess a benzene ring bearing one or more hydroxy groups, including functional derivatives. According to Harborne et al. (1994) phenolic compounds are grouped into the following categories: 1. phenols, phenolic acids, phenylacetic acids; 2. cinnamic acids, coumarins, isocoumarins and chromones; 3. lignans; 4. ten group of flavonoids; 5. lignins; 6. tannins; 7. benzophenones, xanthones, and stilbenes; 8. quinones; 9. betacyanins. Most phenolic compounds are found in nature in a conjugated form, mainly with a sugar molecule. In the case of virgin olive oils, “polyphenols” mostly refers to hydrolysis products of oleuropein and

ligstroside, aglycones, and related compounds. The phenolic fraction of VOO consists of an heterogeneous mixture of compounds, each of which varies in chemical properties and impacts on the quality of VOO (Psomiadou, 2003). The occurrence of hydrophilic phenols in VOO was observed more than 40 years ago by Cantarelli and Montedoro (1969). They established a set of research priorities related to polyphenols which remain practically unchanged to this day:

– Development of an analytical procedure to quantify phenolic compounds in oils.

– Estimation of the levels of phenolic compounds in vegetables oils.

– Possible relationship between these compounds and the characteristics of the olive fruit (variety, degree of ripeness).

– Effect of extraction technology and refining process on the level of polyphenols.

– Importance of phenolic compounds as natural antioxidants.

– Possible role of polyphenols in justifying why olive oils with high peroxide values have considerable stability. It has not been easy to satisfy these points and many researchers are still working on them. However, very interesting systematic studies of the individual classes of hydrophilic phenols in VOO have been developed recently, and it is possible to say that the composition of VOO is today largely elucidated. VOO contains different classes of phenolic compounds such as phenolic acids, phenolic alcohols, flavonoids, hydroxy-isocromans, secoiridoids, and lignans as reported in Table 1.1.

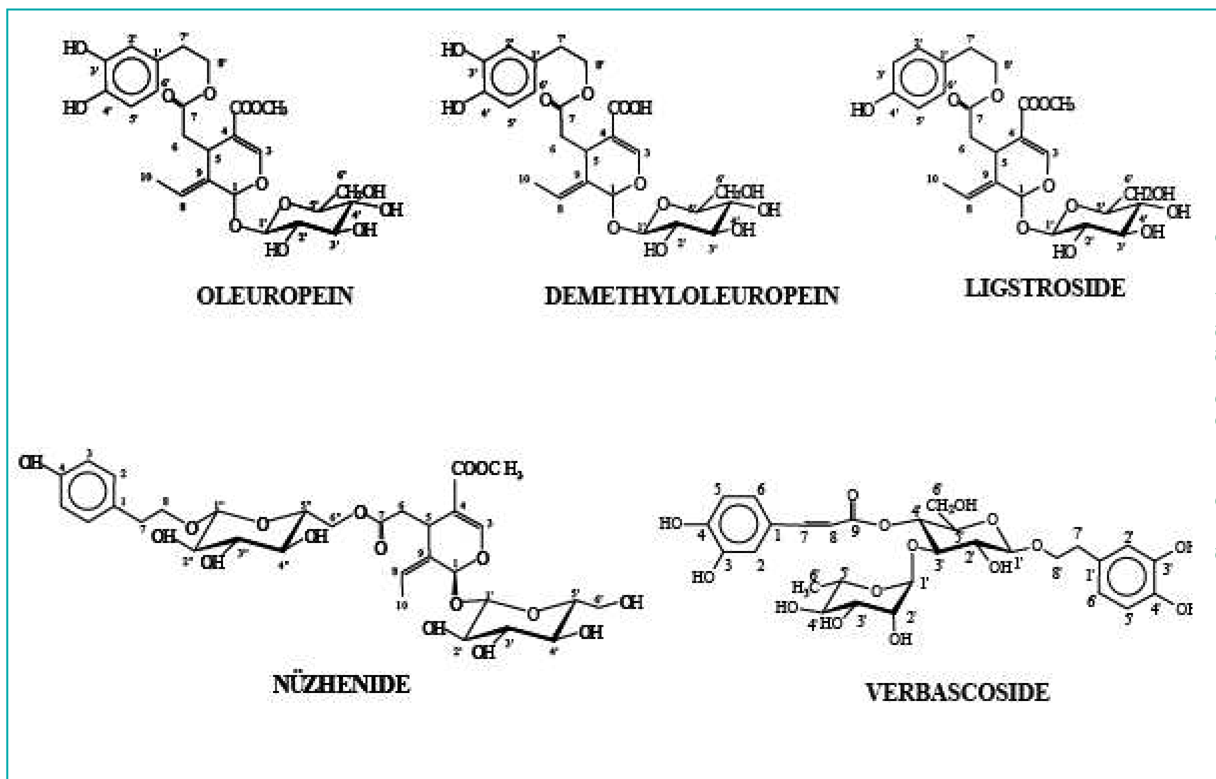
Table 1.1 Phenolic compounds in virgin olive oil and molecular weight

Phenolic acids **Benzoic acid derivates** Gallic acid (170) Gentisic acid (154) Benzoic acid (138) Vanillic acid (168) Protocateutic acid *p*-Hydroxybenzoic acid (138) Syringic acid (198) **Cinnamic acid derivates** Caffeic acid *p*-Coumaric acid (164) *o*-Cumaric acid (164) Ferulic acid (194) Cinnamic acid Cinapinic acid **Other phenolic acid and derivates** 4-(Acetoxyethyl)-1,2- dihydroxybenzene (154) Dopac (3,4-dihydroxyphenil acetic acid) (168) 4-Hydroxy-3-methoxyphenylacetic acid (182) 3-(3,4-Dihydroxyphenyl) propanoic acid (182)	**Hydroxy-isocromans** 1-phenyl-6,7-dihydroxy-isochroman (242) 1-(3'-methoxy-4'-hydroxy)phenyl-6,7-dihydroxy-isochroman) (288)
Phenolic alcohols Hydroxytyrosol (3,4-Dihydroxyphenyl)ethanol (3,4-DHPEA) (154) Tyrosol *p*-Hydroxyphenyl-ethanol (*p*-HPEA) (138) (3,4-Dihydroxyphenil)ethanol –glucoside 2-(4-hydroxyphenyl)ethyl acetate	**Flavonoids** **Flavones** Apigenin (270) Luteolin (286) **Flavanonol** (+)- Taxifolin (304)
Secoiridoids Oleuropein (540.72) Ligstroside aglycon (362) Oleuropein aglycon (3,4 DHPEA-EA) (378) Dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA (3,4-DHPEA-EDA) (304) Dialdehydic form of decarboxymethyl elenolic acid linked to *p*-HPEA (*p*-HPEA-EDA) (320)	**Lignans** (+)-1-Acetoxypinoresinol (416) (+)-Pinoresinol (358) (+)-1-Hydroxypinoresinol (374)

Phenolic acids with the basic chemical structure of C6- C1 (benzoic acids) and C6-C3 (cinnamic acids), such as caffeic, vanillic, syringic, p-coumaric, o-coumaric, protocatechuic, sinapic, and p-hydroxybenzoic acid, were the first group of phenols observed in VOO (Montedodo, 1972; Vasquez Roncero A., 1978) Several authors confirmed the occurrence of phenolic acids as minor components in VOO (Solinas, 1981; Cortesi, 1983)

The prevalent phenols of VOO, however, are the secoiridoids, that are characterized by the presence of either elenolic acid or elenolic acid derivatives in their molecular structure (Garrido Fernandez Diez, 1997). These compounds, e.g., oleuropein, demethyloleuropein, and ligstroside, are derivatives of the secoiridoid glucosides of olive fruits. Breakdown products of two major phenolic constituents of the olive fruit, oleuropein and ligstroside, form the majority of the phenolic fraction. The most abundant secoiridoids of VOO are the dialdehydic form of elenolic acid linked to hydroxytyrosol = (3,4- dihydroxyphenyl)-ethanol or tyrosol = (p-hydroxyphenyl)- ethanol (3,4-DHPEA-EDA or p-HPEA-EDA) and an isomer of the oleuropein aglycone (3,4-DHPEA-EA). For the first time, these compounds were found by Montedoro et al. (1992a, 1992b) who also assigned their chemical structure (Montedoro, 1993)(Fig 1). Later these structures were confirmed by other authors (Angerosa, 1996). Recently, oleuropein and ligstroside aglycone were also detected as minor phenolic components in VOO (Owen, 2000; Perri, 1999). Hydroxytyrosol and tyrosol are the main phenolic alcohols in VOO. It is also possible to find in VOO hydroxytyrosol acetate (Brenes, 1999), tyrosol acetate (Mateos, 2001), and a glucosidic form of hydroxytyrosol (Bianco, 1998). Several authors have reported that flavonoids such as luteolin and apigenin were also phenolic components of VOO [33, 34]. (+)-Taxifolin, a flavanonol, has recently been found in Spanish virgin olive oil (Carrasco Pancorbo, 2004). The last group of phenols found in VOO are the lignans; Owen et al. (Owen, 2000a Owen 2000b) and Brenes et al.(2000) have recently isolated and characterized (+)-1-acetoxypinoresinol, (+)- pinoresinol, and (+)-1-hydroxypinoresinol as the most frequent lignans in VOO.

The structure of the secoiridoid present in olives together with their hydrolysis products most representative in VOO are reported in Fig 1.1.



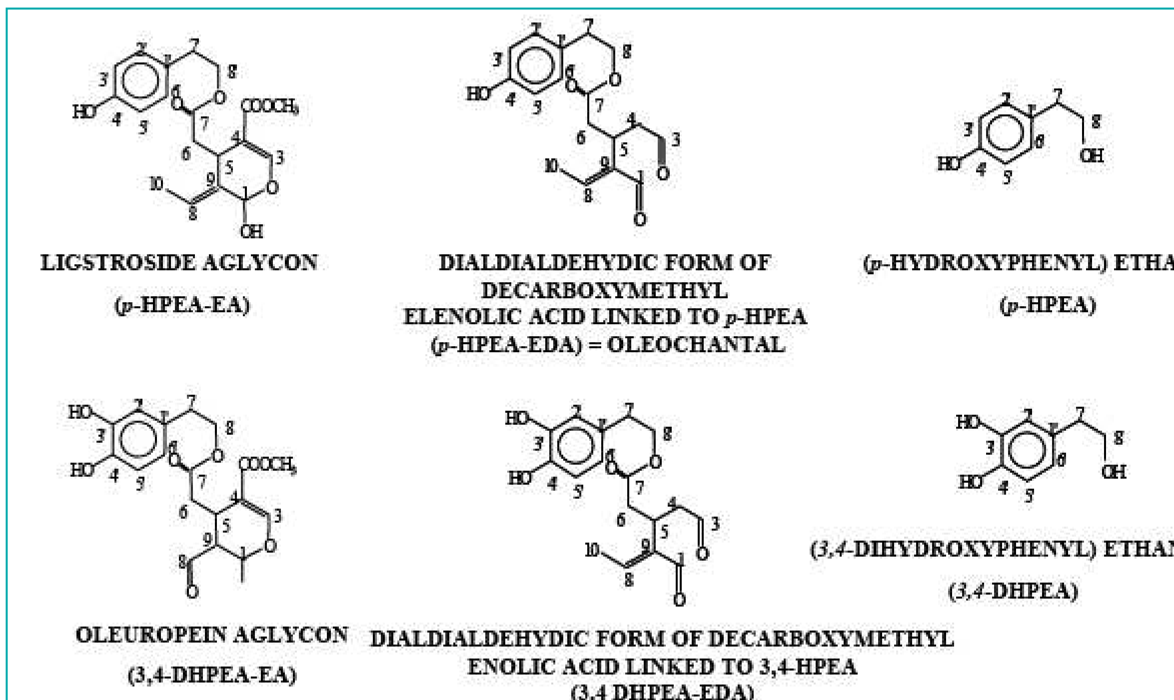


Fig.1.1. Secoiridoids present in olives and their main hydrolysis products in VOO

1.2. Antioxidant properties of biophenols

Oxidation is a process that starts after the VOO has been extracted and leads to deterioration that becomes more pronounced during oil storage.

Natural antioxidants exhibit complex properties between air-oil and oil-water interfaces that significantly affect their relative activities in different lipid systems. The presence of hydrophilic compounds in VVO and their strong antioxidant activity is explained by the so-called "polar paradox" (Poter, 1989) "polar antioxidants are more effective in non polar lipids, whereas non-polar antioxidants are more active in polar lipid emulsions". This means that in a bulk oil system the hydrophilic antioxidants, such as polar phenols are oriented in the air oil interface and become more protective against oxidation than the lypophilic antioxidants, like tocopherols.

A lot of literature results reported that *o*-diphenols, such as 3,4-DHPEA, an the other secoiridoids containing this compound in their molecular structures (DHPEA-EA, 3,4-DHPEA-EDA) are the natural antioxidants of VOO with the highest antioxidant efficiency (Servili 2009).

In a study carried out by Paiva-Martins and co-workers (2006) it was found that when food is processed with VOO in presence of water, olive phenols extracts with higher amount of 3,4-DHPEA-EA and 3,4-DHPEA-EDA would be better than VOO extracts with higher quantities of hydroxytyrosol, despite the higher antioxidant activity of hydroxytyrosol in bulk oil.

Chain-breaking antioxidants, such as phenolic compounds, react with lipid radicals to form unreactive radicals, interrupting the propagation chain. In VOO phenolic compounds exert their antioxidant abilities by scavenging peroxyl and alkoxyl radicals and by chelation of transition metals ions present in trace quantities (Bendini 2007).

Paiva-Martins and Gordon (2005) have studied the antioxidant effects of pure phenolic compounds (Hydroxytyrosol, Hydroxytyrosol acetate, oleuropein, 3,4-DHPEA-EA, 3,4-DHPEA-EDA). The compounds showing the best antioxidant activity in oil-in-water emulsion were 3,4-DHPEA-EA, and 3,4-DHPEA-EDA, which in contrast with oleuropein and hydroxytyrosol did not show pro-oxidant activity. However the radical scavenging activity was measured for these compounds, 3,4-DHPEA-EA showed a much higher activity than 3,4-DHPEA-EDA, suggesting that chelation of iron was of major significance in determining the antioxidant activity of these compounds in presence of iron and water.

Recently, the interest in oxidize forms of VOO phenols has significantly increased, especially in relation to determination of freshness/ageing status (Bendini 2007). Moreover,

cheracterization of these oxidize phenolics could represent an analytical instrument to investigate the thermal processes of the oils during refinement.

Carrasco-Pancorbo and co-workers (2007) studied the phenolic compounds profile after strong heat-treatment (180 °C). The main result in this work was the presence of "unknown" substances, maybe related to phenol oxidation. The concentration of hydroxytyrosol, elenolic acid 3,4-DHPEA-EA and 3,4-DHPEA-EDA decreased more quickly respect to other phenolic compounds present in olive oils, confirming their high antioxidant power.

1.3 Health aspects linked to phenols in VOO

VOO is an integral ingredient of the Mediterranean diet and accumulating evidence suggests that it may have health benefits which include reduction of risk factors of coronary heart disease, prevention of several types of cancers, and modification of immune and inflammatory responses. VOO can be considered as example of functional food, with a variety of components that may contribute to its overall therapeutic characteristics (Bendini 2007, Servili 2009).

The recent epidemiological studies demonstrated that there is a clear association between the consumption of olive oil and reduced risk of cancer in different sites such as breast (Marti-Moreno 1994, Trichopoulou 1995, La Vecchia 1995), prostate (Hodge 2004), lung (Fortes 2003), larynx (Bosetti 2002), ovary (Bosetti 2002) and colon (Stoneham 2000).

Recently there has been a surge in the number of publications that has investigated their biological properties. Bisignano et al. (1999) studied that hydroxytyrosol and oleuropein have antimicrobial activity against several bacterial strains that are causal agents of intestinal or respiratory tract infections in humans. Glatze 1997 et al., demonstrated that enteral immunonutrition with VOO more effectively reduced septic pulmonary dysfunction compared to a fish oil-enriched lipid formula at the same concentration.

Phytochemical compounds such as oleuropein and oleuropein aglycon have been intensively studied for some promising results with respect to human health and their potential medicinal properties. Studies *in vivo* demonstrated that diet containing olive oil phenols may increase resistance of LDLs to oxidation; the effectiveness of oleuropein has been explained in part through its ability to act as an antioxidant and in part through a hypocholesterolemic effect.

Oleuropein aglycon, the bitter component of olives and olive oil, is among the first example on how selected nutrients from a VOO-rich "Mediterranean diet" can directly regulate HER2-driven breast cancer disease (Menendez 2007). As oleuropein aglycon exhibits synergistic anti-tumor effects when concurrently given to breast cancer cells chronically exposed to

trastuzumab (Tzb; Herceptin ™) for several months, this further underscores the potential clinical revelance of these fundings.

The *p*-HPEA-EDA (Fabiani 2008) produced from the hydrolysis of ligustroside, recently called "oleocanthal" has received particular interest because it has been shown to inhibit the activity of both the cyclooxygenase enzymes COX-1 and COX-2, which catalyze steps in the biochemical inflammation pathways derived from arachidonic acid.

This pharmacological effect is similar to that of ibuprofen, a potent modulator of inflammation and analgesia (Beauchamp 2005). Further studies will be necessary to clarify the health promoting activity of the olive oil complex phenolic compounds and these studies will be facilitated when these compounds will be available on the market.

1.4. Organoleptic properties elicited by phenols in VOO

Virgin olive oil is a natural fruit juice obtained only by olives crushing, without any further treatment.

The combined effect of taste, odour (directly via the nose or indirectly through the retronasal path via the mouth) and chemical responses (pungency, astringency, and metallic, cooling and burning) gives rise to a sensation perceived as "flavour". Since sensory quality plays an important role in directing the preference of consumers many attempts have been made to clarify the relationship between the sensory attributes in a VOO as perceived by assessors and its volatile and phenol profiles, which are responsible for aroma and taste, respectively (Caponio 2001, Bendini 2007)

The relationship between organoleptic properties and amount of phenols in VOO has been demonstrated in different works (Servili et al 2004). Phenolic compounds in fact are responsible for bitterness, astringency and pungency of VOO (Servili et al. 2009).

Generally it is assumed that tyrosol, hydroxytyrosol and their derivates are responsible from VOO "pungent" and "bitter".

Garcia et al. (2001) observed that the bitterness of VOO, sensorially measured as well as chemically estimated by the sum of the contents of two secoiridoid derivatives containing hydroxytyrosol (3,4-DHPEA-EA and 3,4-DHPEA-EDA), considered as an objective estimation of this oil taste sensation, decreased clearly according to the temperature used for heating the olives from which it was obtained.

Gutierrez-Rosales et al. (2003) isolated the major peaks present in the phenolic profile using a preparative HPLC; after dissolving in water these molecules purified were then tested to evaluate the intensity of bitterness. In this work a strong correlation between the content of

3,4-DHPEA-EDA, p-HPEA-EDA and 3,4-DHPEA-EA are those mainly responsible for the bitter taste.

1.5. Production of phenolic compounds as defense mechanism in plant

As mentioned before the prevalent secoiridoids in olive oil derive from the hydrolysis of secoiridoids glycosides present in the fruit.

The enzyme responsible for oleuropein hydrolysis is β-glucosidase. In the natural system *in vivo* β-glucosidase is compartmentalized in a different place of their substrate as showed in Fig. 1.2. When olive tissue is damaged, for example during olives crushing, or due to a pathogen attack, the enzyme keeps contact with its substrate and hydrolyzes it.

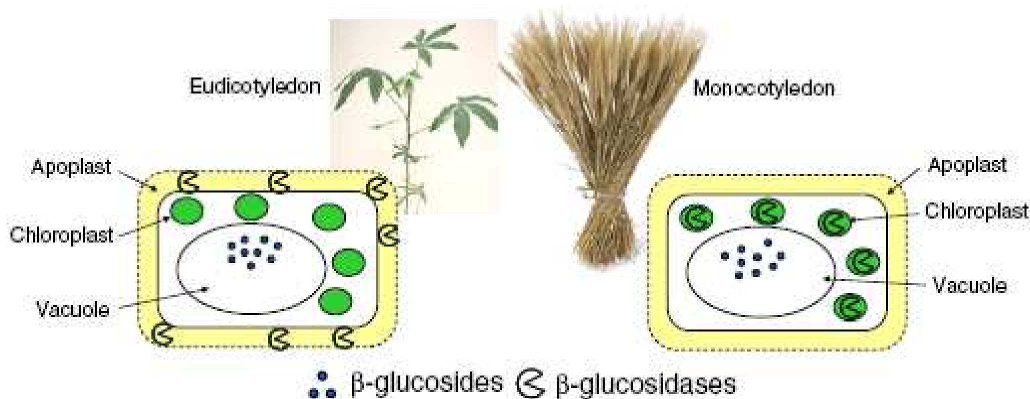


Fig. 1.2. Schematic representation of the different compartmentalization of β-glucosidase and β-glucosides (Source: Morant A.V. et al. Phytochemistry, 69 (2008) 1795-1813)

In plants, β-glucosidases play important roles in diverse aspects of plant physiology, e.g. (1) formation of intermediates in cell wall lignification (Dharmawardhana et al., 1995; Escamilla-Trevino et al., 2006), (2) cell wall degradation in endosperm during germination (Leah et al., 1995) and (3) activation of phytohormones (Kristoffersen et al., 2000; Lee et al., 2006) and (4) activation of chemical defense compounds (Nisius, 1988; Poulton, 1990; Jones et al., 2000; Halkier and Gershenzon, 2006; Suzuki et al., 2006).

In the last period these enzymes are receiving increased attention due to their use in biotechnological and industrial applications; examples are their importance in aroma formation in tea, wine and fruit juice (Mizutani et al., 2002; Maicas and Mateo, 2005; Fia et al., 2005) and in engineering microorganisms for use in biomass conversion as β-glucosidase

constitutes an important part of the cellulase complex (Van Rensburg et al., 1998; Den Haan et al., 2007).

The defence mechanism of plants is different in comparison to animals due to the fact that plants are sessile organisms that cannot flee their predators.

Through the course of evolution, plants have become nature's organic chemists par excellence, and collectively synthesize a plethora of secondary metabolites to defend themselves against herbivores and microorganisms and adapt to different types of abiotic environmental stresses.

The compounds used from defence are divided into two main categories: phytoanticipins and phytoalexins. Phytoanticipins are preformed substances and represents the first barrier to patogens, while phytoalexins are synthetized in response to pathogen attack.

Many plant defense compounds are stored in a non-active glucosylated form to chemically stabilize and increase the solubility of the defense compound, to render it suitable for storage in the vacuole, and to protect the plant from the toxic effects of its own defense system (Jones and Vogt, 2001).

Upon cell disruption, caused for example by a chewing insect, the defense compounds are bioactivated via hydrolysis of the glucosidic linkage catalyzed by β-glucosidases. In intact plant tissue, the β-glucosidases are stored separately from the substrates. This two-component system, of which each of the individual components is chemically inert, provides plants with an immediate chemical defense against attacking herbivores and pathogens.

The catalytic mechanism of β-glucosidase is illustrated in Fig. 1.3.

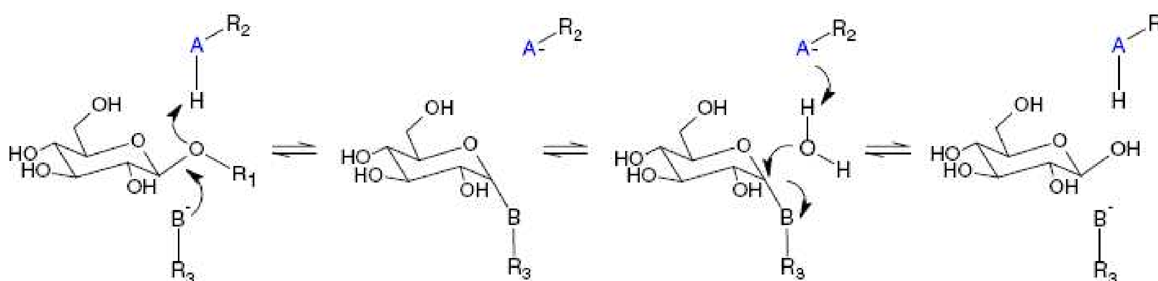


Fig. 1.3. Schematic representation of catalytic action of β-glucosidase (Source: Morant A.V. et al. Phytochemistry, 69 (2008) 1795-1813)

Two conserved glutamic acid residues serve as a catalytic nucleophile and a general acid/base catalyst, respectively. In retaining β-glucosidases, the catalytic glutamic acid residues are situated on opposite sides of the β-glucosidic bond of the docked substrate at a distance of _5.5 Å (Davies and Henrissat, 1995). As the initial step in catalysis, the nucleophile performs

a nucleophilic attack at the anomeric carbon, which results in formation of a glucose–enzyme intermediate. In this process, aglicone departure is facilitated by protonation of the glucosidic oxygen by the acid catalyst. During the second catalytic step (deglucosylation), a water molecule is activated by the catalytic base to serve as a nucleophile for hydrolysis of the glucosidic bond and release of the glucose. Under suitable conditions, β-glucosidases can perform a transglucosylation in which the covalently bound glucose in the enzyme– glucose intermediate is transferred to an alcohol or a second sugar group. The biological activity of the defense compounds is attributed to their hydrolysis products, and therefore separation of glucosides and β-glucosidases into different (sub) cellular compartments in intact tissue is a critical feature of the two component defense systems. As illustrated in Fig.2, the glucosides are stored in the vacuole (Gruhnert et al., 1994) while the subcellular localization of the generally abundantly expressed β-glucosidases differs in monocotyledonous and eudicotyledenous plants. In some plants, the substrate and the bio-activator are additionally separated at the cellular level.

The separate compartmentalization provides the plant with a two-component defense system, in which each separate component is chemically inert. Upon tissue disruption, the glucosides come into contact with the degrading β-glucosidases resulting in an immediate release of toxic defense compounds. Often, the highest amount of the two components is found in seedlings and young plant parts in order to protect the plant from herbivore and pathogen attack at this fragile stage

1.6. Biogenesis of phenolic compounds present in VOO

As explained before the main phenolic compounds present in virgin olive oil such as 3,4-DHPEA, 3,4-DHPEA-EA, 3,4-DHPEA-EDA and are responsible for its antioxidant properties and were originated by the hydrolytic action of β-glucosidases on the glucosides secoiridoids present in olive fruit.

In particular, all the above mentioned compounds are originated from the hydrolysis of oleuropein. Only for the production of 3,4-DHPEA it is necessary the additional action of esterase.

During the crushing of olives the two different compartments (enzyme compartment/ oleuropein compartment) are broken, so the enzyme hydrolyses oleuropein.

To understand the detailed sequence of reactions of oleuropein biotransformation, different works are present in literature that simulates the reaction in batch.

Guiso et al. (2005) characterized the oleuropein hydrolysis products by ^{1}HNMR experiments using two different enzymes, from almond and from olive juice.

As reported in Fig. 1.4 in the first reaction step, by the use of both enzymes the molecules produced are oleuropein aglycon and glucose.

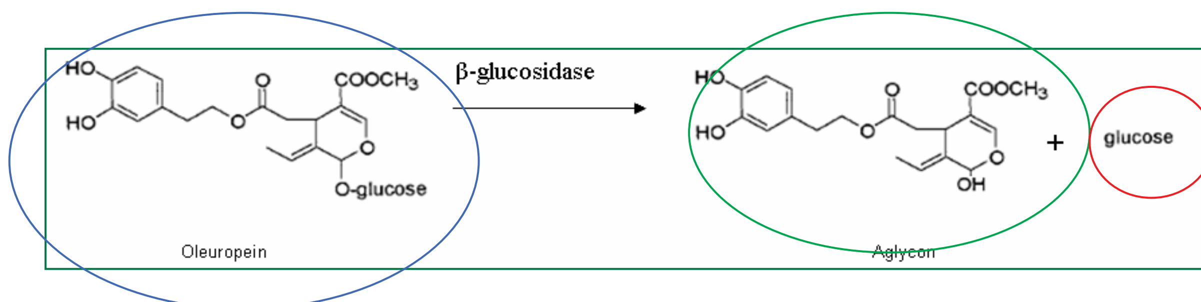


Fig. 1.4. First step of the reaction catalyzed from β-glucosidase

In Fig. 1.5 are also reported the compound present were the reaction is carried out with the commercial enzyme from almond (Bianco et al 2009). The aglycon compound not stable in water is rearranged into water stable compounds, elenolates.

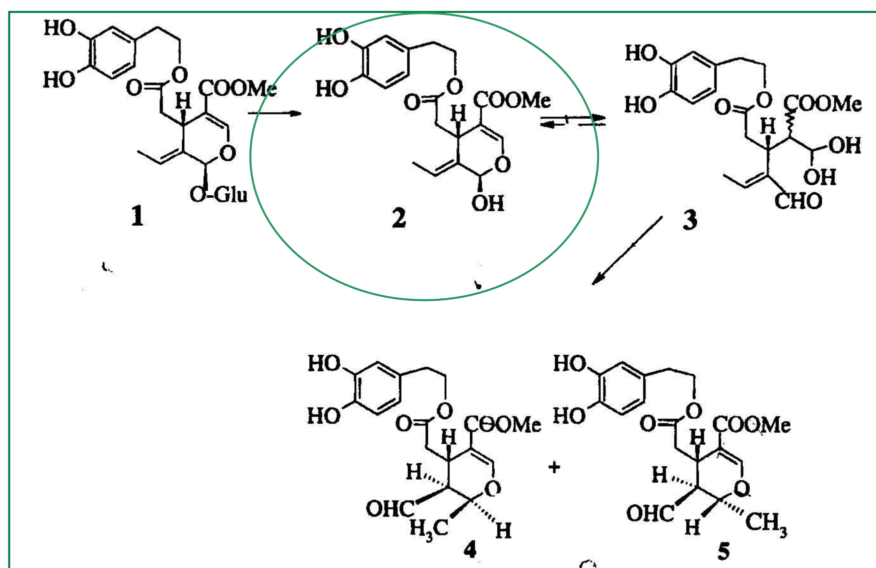


Fig. 1.5. Oleuropein hydrolysis pathway in water phase (Bianco et al 1999)

The absence of the oleuropein aglycon in Olive Mill Waste Water and the high percentage of this compound into VOO is an evidence of the fact that the important antioxidant compound has a different solubility between oil and water. The distribution of 3,4-DHPEA-EA between oil and water is dependent on its solubility in these two phases. Rodis et al (2002) determined

the partition coefficient (K_p= C_{oil}/C_{water}) of oleuropein and its hydrolysis products (3,4-DHPEA, 3,4-DHPEA-EA, 3,4-DHPEA-EDA) between the olive oil and the water, the data obtained are reported in the Table 1.2 In the same table were also reported the partition coefficient of the same compounds changing the organic phase with octanol (Paiva Martins 2002)

Table 1.2 Partition coefficients of oleuropein and its hydrolysis products

Antioxidant	Partition coefficient oil/water	Partition coefficient octanol/water
Oleuropein	0.0006	0.1300
3,4-DHPEA	0.0100	0.0200
3,4-DHPEA-EDA	0.1890	1.0900
3,4-DHPEA-EA	1.4900	1.3800

The highest partition coefficient was found for 3,4-DHPEA-EA showing that this compound has an higher solubility in oil than water.

To mimic and to better understand the complete molecular biotransformation of oleuropein taking place in the olive during fruit ripening or deterioration during crushing and malaxanation, the biphasic liquid system that naturally occur in olives must be considered. Bianco et al (1999) reported the complete sequence of oleuropein metabolites during agrifood processing carrying out the β-glucosidase reaction in water phase and in biomimetic conditions. Biomimetic conditions were created with a biphasic liquid system hydrophilic/lypophilic phases. The reaction mixture in contact with the organic phase allowed the identification of all reaction products, portioned in aqueous or in lypophilic phase at different times. The two phases were then characterized by ^{1}H and ^{13}C NMR analysis, providing the first report of the complete biotransformation of oleuropein with all the intermediated not stable in water. Results obtained by Bianco et al (1999) showed that in the organic fraction, represented by $CDCl_3$, there are the 3,4-DHPEA-EDA and a compounds that increases its concentration during the time that is 3,4-DHPEA-EA as described in Fig. 1.6 where the complete sequence of the oleuropein hydrolysis is reported.

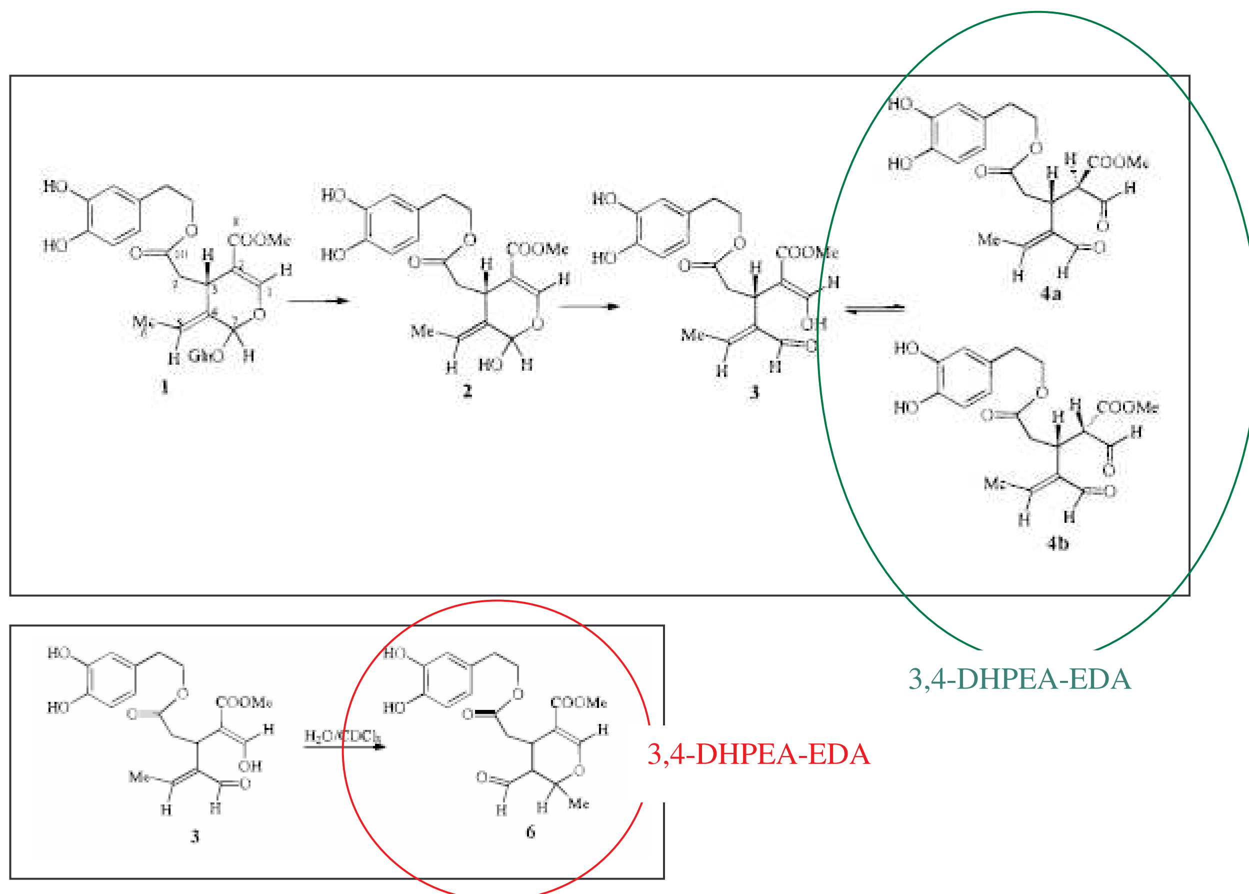


Fig.1.6. Complete sequence of oleuropein biotransformation. Pathway obtained in $CDCl_3$ (Bianco et al. 1999)

The works reported clearly demonstarted that the isomer of oleuropein aglycon present in olive oil is a product from oleuropein biotrasformation but the different phases present in *in vivo* systems cause a partial modification of the initial molecule due to the ket-enolic tautometic equilibrium that involve the ring opening of secoiridoids. The generated isoform of oleuropein aglycon show different chemical physical properties (Garimboldi et al 1986, Angerosa et al. 1995) but maintain the cathecol moiety thus contributing to the antioxidant properties of virgin olive oil (Caruso et al. 2000).

1.7. Renewable material containing phenolic compounds

Olives production is an important agricultural and alimentary sector in Europe. The most important uses of olive fruit is for producing olive oil. The Mediterranean basin is the ain olive oil production area in the world. The annual olive oil production is estimated at about $2{\cdot}10^6$ tons, with Spain, Italy, Greece traditionally being the major producing countries, sharing nearly 80% of the global production.

From olive mill the main by-products are i) olive leaves, which in most case are used by local farmer as animal feed, but are also an important source of antioxidant compounds (oleuropein) ii) olive press cake which is utilized by special oil extracting factories for the production of a lower quality olive oil and for dry olive press cake used as fuel and iii) olive mill waste water (OMWW) which is responsible for the largest environmental problem in oil producing area (Agalias 2007).

For the olive oil production, a considerable amount of water is employed during the continuous washing of olive paste with warm water prior to the process of separation of the oil from the past (malaxation process). The water content depends of the olive oil production methods used, as described in Table 1.3.

Table 1.3 Olive oil production methods

Olive oil production		
	3-phase decanter process	2-phase decanter process
Oil extraction capacity (%)	85	86
Vegetable water (liters/100 Kg of olives)	97.2	8.3
Pomace (Kg/100 Kg of olives)	50.7	72.5

Up to now, a large variety of methods have been suggested for the treatment of waste coming from plant material processing, including: composting, anaerobic digestion, aerobic treatment, mixing with municipal waste water, direct land application, chemical oxidation with combining with biological treatment, adsorption, and even the use of fungi species. However all of the aforementioned methods aim at the decomposition/destruction of the contained polyphenols and not their exploitation.

The treatment of olive oil mill waste (OMWW) is extremely difficult to due to its large volume and increased concentration of organic matter (BOD ranging between 15000-50000 mg/L and COD ranging value as high as about 220 g/L).

The major factor of environmental problems is the high concentration of polyphenols. These compounds if discharged can cause phytotoxicity (Aliotta 2002, Capasso 1992), toxicity against aquatic organisms (Fiorentino 2003) or suppression of soil microorganism (Kotsou 2004) and are difficult to decompose (Davies 2004, Obied 2005).

Although polyphenols can be considered an environmental problem, they are on the other hand, a very important category of antioxidant phytochemicals that are useful for the pharmaceutical and cosmetic industry, as explained in previous paragraphs.

During olive oil production the phenolic compounds present in the olives are distributed between the olive oil (2%) and waste material (98%) further divide in solid waste (45%) and waste water (53 %). An example of the main compounds present in OMWW is reported in table 1.4 (Visioli 1998, 1999)

Table 1.4. Average composition of OMWW (Source: Visioli 1999)

Example of composition of olive mill waste water	
Water (%)	83
Minerals (carbonates, phosphates, potassium and sodium salts etc.) (%)	~ 2
Organic compounds (%)	~ 15
- Sugars	~ 2-8
- Proteins, pectins, macromolecules, etc.	~ 1.2-5
- Polyphenols	~ 0.5-1.8

More than 30 biophenols and related compounds have been identified in OMWW, and the majority of them exhibit antioxidant activity and potential cardio protective and cancer-preventing activities in humans. In table 1.5 the biophenols present in OMWW and their main active properties are reported

Table 1.5. Main biophenols present in OMWW and their bioactivity

Biophenol	Bioactivity
hydroxytyrosol	Antioxidant, cardioprotective and antiatherogenic Chemopreventive, antimicrobial, anti-inflammatory skin bleaching
oleuropein	Antioxidant, antiatherogenic and cardioprotective Hypoglycaemic, antihypertensive, antimicrobial and antiviral anti-inflammatory, cytostatic, endocrinal activity, enzyme modulation
tyrosol	Antioxidant, anti-inflammatory, antiatherogenic, cardioactive
caffeic acid	antioxidant, chemoprotective, antiatherogenic, antimicrobial, anti-inflammatory antidepressive-like activity
vanillic acid	Antioxidant, antimicrobial
verbascoside	Antioxidant, chemoprevention, cardioactive, antihypertensive, anti-inflammatory Antiatherogenic, sedative
elenolic acid	Antimicrobial, antiviral
p-coumaric acid	Antioxidant, antimicrobial, chemoprevention
catechol	Phytotoxic, antimicrobial, carcinogenic activity, antioxidant and anticancer
rutin	Antioxidant, antiatherogenic, anti-inflammatory, chemopreventive

An amount of solid wastes (leaves and small twigs) is produced during the cleaning of the olives prior to milling. Nevertheless, these by-products do not present a management problem

since they can be used as animal feed or as a calorific source. Olive leaves are a well-known source of antioxidant compounds (See Table 1.6) and are marketed as herbal teas with diuretic, antihypertensive and antioxidant effects. Briante et al. (2004) proposed a bioreactor for the production of highly purified antioxidants, which could be converted into pharmacologically active phenolic compounds starting from olive leaves. Turano et al. (2002) proposed an integrated centrifugation–ultrafiltration system that allows the reduction of pollution and the selective separation of some useful products (lipid, sugar, polyphenol). Many other oil waste valorisation methods have been studied: reverse osmosis photochemical degradation of phenols electrocoagulation (Adhoum 2004), etc. Some olive mills are provided with a system able to remove stones from the olive pulp. This process is often used to improve the oil extraction yield. Besides, stones, also called pits, are a valuable product because of their high calorific power. They are currently used as an energy source, but they have been proposed for other interesting uses such as soil-less substrate for hydroponics and to produce activated carbon. Moreover, Fernández-Bolaños (2001) proposed steam-explosion of olive stones to obtain high-value-added products such as hydroxytyrosol xylo-oligosaccharides and high quality lignin. Furthermore, Montané (2002) produced furfural through high-temperature acid hydrolysis of olive stones (Roig 2006).

Table 1.6 Example of phenolic compounds composition extracted from olive leaves (source Roing et al. 2006)

Phenolic compounds	% Absolute (content dry basis)
Hydroxytyrosol	1.46
Tyrosol	0.71
Catechin	0.04
Caffeic acid	0.34
Vanillic acid	0.63
Vanillin	0.05
Rutin	0.05
Luteolin-7-glucoside	1.38
Verbascoside	1.11
Apigenin-7-glucoside	1.37
Diosmetin-7-glucoside	0.54
Oleuropein	24.54
Luteolin	0.21
Diosmetin	0.05

References

Adhoum, N., Monser, L.,. Chemical Engineering and Processing 43 (2004),1281–1287.

Agalias A., Magiatis P., Skaltsounis A., Mikros E., Tsarbopoulos, Gikas E., Spanos I., Manios T., J J.Agric. Food Chem 55 (2007) 2671-2676

Aliotta G., Fiorentino A., Oliva A., Temussi F., Alleopathy J. 9 (2002) 9-17

Angerosa F., D'Alessandro N., Konstantinou P., Di Giacinto L., J. Chromatogr. A 736 (1996)195–203.

Angerosa F., d'Alessndro N., Corana F., Mellerio G., J. Chromatography 736 (1996) 195-203

Beauchamp, G. K., Keast, R. S. J., Morel, D. Nature, 437 (2005) 45–6.

Bendini A., Cerretani L., Carrasco-Pancorbo A., Gómez-Caravaca A.M., Segura-Carretero A. , Fernández-Gutiérrez A., Lercker G. Molecules 12, (2007) 1679-1719

Bianco A. D., Piperno A., Romeo G., Uccella N., J. Agric. Food Chem., 47 (1999), 3665-3668.

Bianco A.D., Muzzalupo L., Romeo G., Scarpati M.L., Soriero A., Uccella N., Food Chem. 63 (1998) 461–464.

Bisignano, G. Tomaino, A. Lo Cascio, R. Crisafi, G. Uccella, N. Saija, A. J. Pharm. Pharmacol. 31 (1999) 971–974.

Bosetti, C., Negri, E., Franceschi, S. Cancer Causes Control, 13, (2002) 465–70.

Brenes M., Garcia A., P. Garcia, J.J. Rios, A. Garrido, J.Agric. Food Chem. 47 (1999) 3535–3540.

Brenes M., Hidalgo F.J., Garcia A., Rios J.J., Garcia P., Zamora R., Garrido A., J. Am. Oil Chem. Soc. 77 (2000) 715–720.

Briante, R., Patumi, M., Febbraio, F., Nucci, R.,. Journal of Biotechnology 111 (2004) 67–77.

Capasso R., Cristinzio G., Evidente A., Scognamiglio F., Phytochemistry 31 (1992) 4125-4128

Caponio, F.; Gomes, T.; Pasqualone, A. Eur Food Res. Technol., 212 (2001) 329–333

Carrasco Pancorbo A., Cruces-Blanco C., Segura Carretero A., Fernandez Gutierrez A., J. Agric. Food. Chem. 52 (2004) 6687–6693.

Carrasco-Pancorbo, A.; Cerretani, L.; Bendini, A.; Segura-Carretero, A.; Lercker, G.; Fernández-Gutiérrez, A. J. Agric. Food Chem. 55(2007) 4771-4780.

Caruso D., Colombo R., Patelli R., Giavarini F., Galli G., J.Agric. Food Chem. 48 (2000) 1182-1185.P.,

Cortesi N., Fedeli E., Riv. Ital. Sostanze Grasse 1983, 60, 341–351.

Davies, G., Henrissat, B., Structure 3, (1995) 853–859.

Davies L.C., Vilhena A.M., Novais J.M., Martins-Dias S., Grasas Aceites 55 (2004) 233-241
Den Haan, R., Rose, S.H., Lynd, L.R., van Zyl, W.H.,. Metab. Eng. 9 (2007) 87–94.
Dharmawardhana, D.P., Ellis, B.E., Carlson, J.E., Plant Physiol. 107(1995) 331–339.
Escamilla-Trevino, L.L., Chen, W., Card, M.L., Shih, M.C., Cheng, C.L., Poulton, J.E., Phytochemistry 67 (2006) 1651–1660.
Fabiani, R., Rosignoli, P., De Bartolomeo, A.138, (2008). 1411–6.
Fernandez-Bolanos, J., Rodriguez, G., Rodriguez, R., Heredia, A., Guille´n, R., Jime´nez, A., J. Agric. Food Chem. 50 (2002) 6804–6811.
Fia, G., Giovani, G., Rosi, I., J. Appl. Microbiol. 99 (2005) 509–517.
Fiorentino A., Gentili A., Isidori M., Monaco P., Nardelli A., Parrella A., Temussi F., J.Agric. Food Chem. 51 (2003) 1005-1009
Fortes, C., Forastiere, F., Farchi, S. Nutr. Cancer, 46 (2003) 30–7.
García, J. M., Yousfi, K., Mateos, R. et al. J. Agric. Food Chem. 49 (2001) 4231–5.
Garimboldi P., Jommi G., Verotta L., Phytochem 25 (1986) 865-869
Garrido Fernandez Diez M.J., Adamos M. R., Table olives. Chapman & Hall, London (UK), 1997, pp. 67–109.
Glatzle, J.; Beckert, S.; Kasparek, M. S.; Mueller, M. H.; Mayer, P.; Meile, T.; Konigsrainer, A.; Steurer, W. Langenbecks Arch Surg, 392 (2007) 323–329
Gruhnert, C., Biehl, B., Selmar, D., Planta 195 (1994) 36–42.
Guiso M., Marra C. Nat. Prod. Res. 19 (2005) 105-109
Gutierrez-Rosales, F.; Rios, J. J.; Gomez-Rey, MA. L. J. Agric. Food Chem. 51 (2003) 6021-6025.
Halkier, B.A., Gershenzon, J., Annu. Rev. Plant Biol. 57 (2006) 303–333.
Harborne J.B. The Flonoids: Advances in Research since 1986; Chapman and Hall: London, U.K 1994
Hodge, A. M., English, D. R., McCredie, M. R. et al. (2004). Cancer Causes Control, 15, 11–20.
J. Agric. Food Chem. 2005, 53, 2704-2709.
Jones, P.R., Andersen, M.D., Nielsen, J.S., Høj, P.B., Møller, B.L., 2000.. In: Romeo, J.T., Ibrahim, R., Varin, L., De Luca, V. (Eds.), Evolution of Metabolic Pathways. Elsevier Science Ltd., New York, pp. 191–247.
Jones, P., Vogt, T., Planta 213 (2001)164–174.
Kotsou M., Mari I., Lasaridi K., Chatzipavlidis I., Balis C., Kyriacou A., Appl. Soil Ecol. 26 (2004) 113-121.

Kristoffersen, P., Brzobohaty, B., Hohfeld, I., Bako, L., Melkonian, M., Palme, K., 2000. Planta 210, 407–415.

La Vecchia, C., Negri, E., Franceschi, S Cancer Causes Control,.6, (1995). 545–50.

Leah, R., Kigel, J., Svendsen, I., Mundy, J., J. Biol. Chem. 270 (1995)15789–15797.

Lee, K.H., Piao, H.L., Kim, H.Y., Choi, S.M., Jiang, F., Hartung, W., Hwang, I., Kwak, J.M., Lee, I.J., Hwang, I.,. Cell 126 (2006) 1109– 1120.

Maicas, S., Mateo, J.J., Appl. Microbiol. Biotechnol. 67 (2005) 322–335.

Martin-Moreno, J. M, Willett, W. C, Gorgojo L, Int. J. Cancer, 58, (1994). 774–780.

Mateos R., Espartero J.L., Trujillo M., Rios J.J., Leon-Camacho M., Alcudia F., Cert A., J. Agric. Food Chem. 49 (2001) 2185–2192.

Menéndez, J. A.; Vázquez-Martín, A.; Colomer, R.; Carrasco-Pancorbo, A.; García-Villalba, R.; Fernández-Gutiérrez, A.; Segura-Carretero, A. BMC Cancer 2007, 7, 80. DOI: 10.1186/1471-2407-7-80.

Mizutani, M., Nakanishi, H., Ema, J., Ma, S.J., Noguchi, E., Inohara-Ochiai, M., Fukuchi-Mizutani, M., Nakao, M., Sakata, K., 2002. Plant Physiol. 130, 2164–2176.

Montane´, D., Salvado´ , J., Torras, C., Farriol, X., Biomass and Bioenergy 22 (2002)195–304.

Montedoro G.F, Cantarelli C., Indagini sulle sotanze fenoliche presenti negli oli d'oliva. Riv. Ital. Sost. Grasse, 46, (1969) 115-124.

Montedoro G.F., Sci. Technol. Aliment. , 3 (1972) 177–186.

Montedoro G.F., Servili M., Baldioli M., Miniati E., J. Agric. Food. Chem. 40 (1992) 1571–1576.a

Montedoro G.F., Servili M., Baldioli M., Miniati E., J. Agric. Food. Chem. 40 (1992) 1577–1580.b

Montedoro G.F., Servili M., Baldioli M., Selvaggini R., Miniati E., Macchioni A., J. Agric. Food. Chem. 41 (1993) 2228-2234

Nisius, A., Planta 173, (1988) 474– 481.

Obied H.K., Allen M.S., Bedgood D.R., Prenzler P.D., Robards K., Stockmann R., J.Agric. Food Chem 53 (2005) 823-837

Owen R.W., Mier W., Giacosa A., Hull W.E., Spiegelhalder B., Bartsch H., Food Chem. Toxicol. 38 (2000) 647– 659.a

Owen R.W., Mier W., Giacosa A., Hull W.E., Spiegelhalder B., Bartsch H., Clin. Chem. 46 (2000) 976–988.b

Paiva Martins F., Gordon M.H., JAOCS 79, (2002) 571-576

Paiva-Martins, F.; Santos, V.; Mangericao, H.; Gordon, M. H.. J. Agric. Food Chem. 54 (2006) 3738-3743.

Perri E., Raffaelli A., Sindona G., J. Agric. Food Chem. 47(1999) 4156–4160.

Porter, W.L.; Black, E.D.; Drolet, A.M. J.Agric. Food Chem. 37(1989) 615-624.

Poulton, J.E., Plant Physiol. 94, (1990) 401–405.

Psomiadou E., Konstantinos X., Blekas K.G, Tsimidou, M.Z., Boskou D., Eur. J. Lipid Sci. Technol. 105 (2003) 403–408

Roig, A., Cayuela M.L., Sánchez-Monedero M.A., Waste Management 26 (2006) 960-969

Servili, M., Selvaggini, R., Esposto, S. et al. J. Chromatgr. 1054 (2004) 113–27.

Servili M., Esposto S., Fabiani R., Urbani S., Taticchi A., Mariucci F., Selvaggini R., Montedoro G. F. Inflammopharmacology 17 (2009) 76–84

Solinas M., Cichelli A., Riv. Ital. Sostanze Grasse 58(1981) 159–164.

Stoneham, M., Goldacre, M., Seagroatt, V. et alJ. Epidemiol. Community Health. 54 (2000) 756-60.

Suzuki, H., Takahashi, S., Watanabe, R., Fukushima, Y., Fujita, N., Noguchi, A.,Yokoyama, R., Nishitani, K., Nishino, T., Nakayama, T., J. Biol. Chem. 281 (2006) 30251–30259.

Trichopoulou, A., Katsouyanni, K., Stuver, S., J. Natl. Cancer Inst. 82, (1995). 110–6.

Turano, E., Curcio, S., De Paola, M.G., Calabro` , V., Iorio, G., Journal of Membrane Science 209 (2002) 519–531.

Van Rensburg, P., van Zyl, W.H., Pretorius, I.S., Yeast 14 (1998)67–76.

Vazquez Roncero A., Rev. Fr. Corps Gras. 25(1978) 21–26.

Visoli F., Bellomo G., Galli C., Biochem. Biophys. Res. Commun. 247 (1998) 60-64

Visioli F., Romani A., Mulinacci N., Zarini S., Conte D., Vincieri F.F., Galli C., J.Agric. Food Chem 47 (1999) 3397-3401.

Bioartificial membrane systems biomimiking *in vivo* processes

Chapter 2

Clustering of interactions between bioactive macromolecules and membranes, and identification of resulting properties of the nanostructured biohybrid membrane systems

2.1 Introduction

In living systems, interactions between biomolecules and membranes play a critical role in the regulation of processes such as transformations, transport, communication. As for living systems, the development of efficient artificial biohybrid systems relies on the thorough understanding of the molecular mechanisms of bio/non-bio interactions in order to predict, promote and control events occurring on the artificial membrane surface and through its thickness. A huge amount of information, coming from different disciplines, is present in the literature on biological macromolecules interactions with other molecules, biological membranes, surfaces. A clustering of these information is needed in order to achieve a shared base among the various disciplines in order to promote significant advances.
The aim of this document is to identify the aspects of major interest in membrane/biomolecule interaction for the development of nanostructured biohybrid membrane systems. The attention has been mainly focused on proteins as model biomacromolecules.
The interaction of proteins with membranes plays a central role in many biotechnological processes. Although there are many different types of membranes, which interact with many different proteins under very different circumstances, there are great similarities between all these interactions. Hence, understanding the biophysics of protein-membrane interaction is crucial for understanding phenomena which may permit to improve the processes in which an interaction between macromolecules and membranes is needed.
Protein-membrane interaction is involved biohybrid membranes preparation, biofunctional membrane, in protein purification or fractionation, macromolecules heterogenization for biocatalytic membrane reactors, biosensors, in membrane emulsification process where protein is used as emulsifier, and in affinity membranes.
When a stable bound between macromolecules and membranes is needed (e.g. biocatalytic membrane reactor) it is necessary taking into account that the interaction membrane/molecule

do not have to modify the 3D structure of the biocatalyst. In the processes in which any interaction between membrane and macromolecules is needed (e.g. protein fractionation) it necessary to minimize biofouling phenomena.

Physicochemical characteristics of both biomolecule and membrane and fluid-dynamic conditions are the main factor to be considered in the development of membrane bioprocesses.

Some of these properties to be considered are:

- Shape and nature of the targeted species
- pH and Ionic strength
- Membrane characteristics

The interaction between protein and membrane is influenced from the complexity of protein molecular structure and properties. Protein molecules are multi-polar with multiple charge point and the overall net charge depends on the solution pH. The active size depends on the ionic strengths or salt concentration, as protein molecules interact with ions and water molecules. The ionic strength affects the interactions between protein molecules and hence the solubility. This complexities make it difficult to derive an adapt protocol to predict all the kind of protein/membrane interactions.

2.2 Protein properties

Before protein adsorption behaviour can be understood, it is first important to understand the basic makeup of protein structure. Proteins are complex copolymers that are made up of four levels of structure, designated as the primary, secondary, tertiary, and quaternary structures.

Few proteins and molecules are spherical in shape, consequently their passage through the membrane is strongly affected by their random alignment relative to the pores in the membranes resulting in a statistical results rather than an absolute retention value. In presence of complex protein solution also the nature of different components can affect the passage/retention of the individual species. Some may have different solubility; consequently the concentration at the membrane surface can lead to precipitation and change the apparent retention characteristic of the system. pH and ionic strength solution in which the biomolecule is immersed can affect the protein shape and size. Consequently also the retention of protein of a particular protein/membrane system may change properly. A very common example is

the case of 64 kDa BSA that can behave a molecule of 300 kDa in a particular case of low ionic strength and pH significantly different for the ionic point (Zydney 1998).
Table 2.1. provides some characteristics of proteins involved in food process.

Table 2.1. Molecular weight and isoelectric point of biomolecules

Biomolecules	**Molecular weight**	**Isoelectric pH**
Cytocrome c	12,200	10.4
Pepsin	35,500	< 1.0
α-casein	30,000	4.1
Myoglobine	17,500	7
Ovalbumin	40,000	4.6
α- Chymotrypsinogen	18,000	9.6
Bovine serum albumin (monomer)	69,000	4.7
Bovine serum albumin (dimer)	134,000	4.8
Pulmozyme	43,000	4.5
α-Lactalbumin	14,400	4.2
β-Lactoglobulin	18,300	5.3
Ovotransferrin	80,000	5.5
Immunoglobulin G	155,000	6.6
Hemoglobin	67,000	7.1
Ribonuclease A	13,700	7.8
Lysozyme	14,300	11.0

The general model used to describe the protein adsorption to a surface depends on protein concentration in solution and concentration of available surface for adsorption (Latour 2005). The adsorbed protein then may transition to an irreversibly adsorbed state as a result of orientation or conformational changes that further reduce the free energy of the system. A surface that adsorbs a protein in a manner that includes this part of the process will inevitably become saturated with the adsorbed protein if sufficient interaction time is provided. The state of the final adsorbed protein layer will be determined by the manner in which the proteins are able to organize themselves on the surface, which is influenced by both protein–surface and protein–protein interactions in the presence of the surrounding aqueous solution. The proteins that adsorb to the surface in the latter stages of this process will tend to be increasingly inhibited from the irreversible step of this process by steric restrictions caused by the

previously adsorbed proteins. Because of this, the final fraction of the adsorbed protein layer is more likely to be maintained in its native and possibly reversibly adsorbed state.

2.3 Techniques for basic make-up of protein characterization

Physical protein-protein interaction have been studied extensively because of their crucial role in controlling central biological processes such as cell division and their implication in a range of human diseases including cancer. A collection of experimental techniques is available to characterize protein-protein interactions; some of them are most suitable to determine stable complexes and others are generally considered to detect transient interactions.

Some techniques are useful to characterize the biomolecule in terms of structural and conformational properties.

Small angle X-ray scattering (SAXS) provides rapid, conformational analysis of macromolecules in solution. SAXS can characterize shape and conformation in solution for quite small to very large macromolecular systems, so it avoids the size limitations. In addition, information derived from SAXS data can be useful both prior to and after high resolution structures are solved. SAXS is not only likely to be more powerful in conjunction with atomic resolution structures but is also able in such combinations to provide more accurate and complete models of protein, RNA, and DNA structures, conformations, interactions, and assemblies in solution. The accessible experimental resolution can thus be made appropriate to the biological question being asked with SAXS measurements directly defining global shape and conformation in solution, whereas the combination of SAXS with computation plus high-resolution component structures provides more detailed three-dimensional information.

Magic-angle spinning solid state NMR (MAS SSNMR) has been shown recently to be a complementary and successful method for studying the structure and dynamics of biological macromolecules. Examples are membrane proteins, protein aggregates and fibrils, and recently, the Pf1 filamentous phage. Also, sample preparation for MAS SSNMR is more flexible, and measurements can be performed in conditions, which are either biologically relevant, or important for their biophysical characterization.

2.4 Membrane properties

Essentially also all membranes acquire a net surface change in aqueous solution due to the adsorption of specific positive or negative ions from the aqueous solution and or the ionization of specific chemical group on their surface (e.g. the ionization of carboxylic acid groups yields $–COO^-$ anions). Most membranes at neutral pH, but also macromolecules, have a net negative charge because of the negative adsorption of negative ions. The magnitude of negative charge increases in alkaline solution due to an increase in anion adsorption, an increase in ionization of any acidic groups (e.g. $-COOH \rightarrow -COO^-$), and /or the deionization (deprotonation) of any basic groups (e.g. $–NH_3^+ \rightarrow NH_2$). The reverse behaviour is true in acidic solution. Thus, as the solution pH is lowered, most amphoteric species will eventually pass through a point in which they have no net charge, with the pH at which this occurs referred to the isoelectric point or PI (Zeman and Zydney, 1996). In the Table 2.2 the most common membrane used for protein treatment and its iso-electric point is reported.

Table 2.2.

Membrane material	isoelectric pH	References
Aluminum oxide	4.4	Bowen W.R. 1990
Polysulfone	3.6	Nystrom M 1989
Polycarbonate	2.5	Keesom W.H. 1988
Sulfonated polysulfone	<2.5	Nystrom M 1989
Cellulose acetate/nitrate	2.2	Bowen W.R. 1984
Polyethersulfone	3.1	Pontié M . 1997
Polysulfone	0.5	Pontié M . 1997
SPG	1.5	Ryosuke T. 1996

The electrostatic nature of both membrane and macromolecules determines attraction and repulsion phenomena. If the membrane and the biomolecule have the same electrostatic charge, repulsion naturally occurs. If the membrane surface has an opposite charge of the protein molecule, the surface will attract a monolayer of charged protein molecules, to form a self repulsive layer. Therefore, if the convective flows overcomes the electrostatic repulsion the protein molecules can pass through, while otherwise it is retained (Cui 2005). An added complexity is the influence of ionic strength on the charges of the proteins and membrane surface. Ionic strength can decrease the electrostatic effect shielding the charge present on

both membrane and protein but low ionic strength can cause low protein solubility facilitating protein aggregation.

The other interaction that can occur between membrane surface and protein molecules are hydrophobic and van der Waals. Proteins are strongly hydrophobic at their iso-electric point and their adsorption on membranes may therefore be increased due to hydrophobic interaction.

The choice of membrane characteristics is also an important aspect in the protein –membrane interaction. Parameters to be considered are: pore size, pore size distribution, porosity, surface porosity, wettability and retention measurement.

The study and the analysis of membrane surfaces involves both physical and chemical techniques.

Several modern methods for the membrane probe the topmost 1–10 nm of the surfaces exposed to vacuum. These include X-ray photoelectron spectroscopy, Auger electron spectroscopy, low-energy electron diffraction, electron energy loss spectroscopy, thermal desorption spectroscopy, ion scattering spectroscopy, secondary ion mass spectrometry.

Membrane surface chemistry creates very important properties such as hydrophilicity or hydrophobicity (relates to surface free energy), presence of ionic charges, membrane chemical or thermal resistance, binding affinity for particles in a solution, and biocompatibility (in case of biomedical applications). Hydrophilicity and hydrophobicity of membrane surfaces can be expressed in terms of water (liquid) contact angle θ. The presence of the charges changes the properties of membrane-liquid interface. Membrane surface may, therefore, develop corresponding electrochemical potential and induce the formation of the layers of solution particles trying to neutralize the charges.

2.5 Technologies to understand protein-membrane interaction

The analysis of interactions between protein-protein and membrane –protein interaction require data on interfacial concentration, protein conformation, and dynamic behaviour of proteins at interfaces. Some experimental techniques are particularly adapted for adsorption studies, on surfaces.

Small angle neutron scattering (SANS) reflectometry, and ellipsometry help in characterization *in situ* the orientations of ellipsoid protein at interfaces.

Atomic force microscopy (AFM) can provide the spatial distributions of a smooth surface, and can produce images at molecular scale resolution by a scanning a microscopic tip at the

end a cantilever over a surface. Uniquely, AFM can image surfaces in air or liquid without any special sample preparation. This technique provides informations about surface morphology, surface porosity and the dimensions of adsorbed agglomerates. Another key feature of AFM is its ability to measure force interactions as a functions of probe-surface separation distances (Bowen 2002). One area of significant progress is the imaging of nucleic acids. The ability to generate nanometer-resolved images of unmodified nucleic acids has broad biological applications. Chromosome mapping, transcription, translation and small molecule-DNA interactions such as intercalating mutagens, provide exciting topics for high-resolution studies. The first highly reproducible AFM images of DNA were obtained only in 1991. Four major advances that have enabled clear resolution of nucleic acids are: Control of the local imaging environment including sample modification; TappingMode scanning techniques; Improved AFM probes (such as standard silicon nitride probes modified by electron beam deposition and Oxide Sharpened NanoProbes) and Compatible substrates (such as salinized mica and carbon coated mica). The recent innovation, such as Digital Instruments BioScope system, which combines the high resolution of AFM with the ease of use and familiarity of inverted optical microscopes, has further added to the attractiveness of AFM for biological imaging. Bright-field, fluorescence and other optical techniques can be used to identify structures of interest while the AFM simultaneously generates nanometer-resolved images of the sample surface.

In addition to interfacial concentration of biomolecules, the total internal reflection fluorescence (TIRF) technique with fluorescent labels sensitive to electrical potential gives some clues about orientation of adsorbed proteins. This is possible when Debye screening length and protein size are of the same order of magnitude. Attenuated total reflection-Fourier transform infrared absorption (ATR-FTIR), is helpful to characterize intermolecular interaction and protein conformational changes at flat interfaces with respect to their state in solution, like the variation of the α-helix and β-sheet contents (Balme 2006).

Paramagnetic species may interact via dipolar mechanisms. The strength of this interaction is governed by $(\text{distance})^{-2}$ functionality. These phenomena produces changes in the lineshape and intensities on the electrotro paramegnetic (EPR) spectra. EPR spectroscopy is based on spin-spin interaction of protein bound spin-labels that causes dipolar broadening of the EPR spectra. This line broadening is related to spin-label distance and was subsequently used to infer protein conformational changes. This technique can be used to identify tertiary conformational changes of protein after protein-membrane interaction.

Solid-state nuclear magnetic resonance (ssNMR) is a technique to study peptide–surface interactions to enable the distance between isotopically labelled atom types to be measured with subangstrom resolution (Shaw 2000, Bower 2005). By the careful selection of which atoms are labeled in the systems, it is possible to quantitatively determine the secondary structure of adsorbed peptides and to identify the types of peptide residues that specifically interact with functional groups of the surface for several different types of surfaces. This method thus enables specific interactions governing the structure of adsorbed peptides and their associated surface functional groups to be targeted and directly probed.

Circular dichroism spectropolarimetry (CD) has also been demonstrated by several groups to be able to quantitatively determine the secondary structure of adsorbed peptides and proteins, as well as provide information regarding the adsorbed tertiary structure for proteins containing tryptophan residues (Vermer 2000, Lundqvist 2004). In particular, the high sensitivity of this method has enabled its use to be extended to proteins adsorbed onto flat surfaces in addition to particles in solution, thus expanding its versatility to a much broader range of materials while also avoiding issues related to signal noise problems due to light scattering by dispersed solid particles. 2D NMR provides an excellent technique to probe the effect of adsorption on protein structure because Hydrogen/deuterium (H/D) exchange is directly related to the degree of solvent accessibility of a given peptide residue (Engel 2004).

Static time-offlight secondary ion mass spectrometry (ToF-SIMS), can be used to probe adsorbed protein orientation and conformation using ion beam to release molecular fragments from the top 1.0–1.5nm of an adsorbed protein layer (Tidwell 2001, Wagner 2004)

Traditional techniques for biological characterization in molecular biology such as, protein quantification, *in situ* hybridation, western blot analysis, fluorescence protein labelling, mutation studies can be also applied to study the interaction between protein-membrane interaction.

Crespo et al (1999) used fluorescence labelling of protein to monitor protein fractionation and to control fouling phenomena during ultrafiltration process.

Sousa et al. (2001) evaluated the distribution along hollow fibres membranes of the amount of enzyme immobilized on microporous nylon membrane. The procedure was based on measuring the protein content in membranes pieces sectioned after enzyme immobilization, along the module length, i.e. initial, middle and end of the membrane module, by Lowry assay.

Liu et al. (2005) proposed a method for the localization of active peroxidase immobilized on microfiltration flat membranes by the use of 3,3’-Diaminobenzidine tertahydrochloride

(DAB). It can form an insoluble brown polymeric non-droplet precipitate which is strongly osmium-philic when oxidized in presence of H_2O_2. The membranes pieces, after the reaction with DAB, embedded in Epon resin, were cut by ultra-microtome. The visualization of the protein on membrane surface was carried out by electron microscopy.

A combined method (Mazzuca et al 2006) merged from classic *in situ* enzyme activity assay and western blot technique is reported to localize the sites of enzyme immobilization and to determine its catalytic activity on a polymeric capillary membrane reactor.

McGuire and coworkers (Matthews 1995) have used the mutated lysozyme to elucidate relationships between protein stability, adsorption behaviour, and adsorbed-state bioactivity.

Advances in computational hardware, software, and analysis methods, coupled with the structural determination of thousands of proteins that are now available via the Protein Data Bank (PDB), have now provided the capability to conduct all-atom molecular simulations to investigate the atomic level behaviour of proteins in solution.

While these capabilities have been widely developed and exploited for application to the study of protein folding and ligand–receptor interactions for rational drug design, they represent a largely untapped resource for assisting in the understanding of protein adsorption behaviour on biomaterial surfaces (Latour 1999). Latour and colleagues have been working over the past several years to develop the necessary methods to enable molecular modelling to be accurately applied to simulate protein–surface interactions (Agashe 2005, Sun 2005). Jiang and co-workers represented the protein domains of immunoglobulins (IgGs) as connected spheres; this treatment successfully predicted the orientation of experimental IgG adsorption on the surface of varying charges (Chen 2003, Sheng 2002).

Several researchers are exploring detailed atomic representations of proteins. The earliest studies to use protein crystal structures to simulate the adsorption process assumed a completely rigid protein and calculated screened coulomb and Lennard–Jones interactions over all protein rotations and distances. Ravichandran et al. (2001) have similarly explored rigid atomistic models with electrostatic treatments, and found that a net positively charged protein (lysozyme) could adsorb on a positively charged surface, due to the non uniformity of the charge distribution on the protein. Zhou et al. (2003) predicted the orientations of an adsorbed antibody on a surface using a united residue model, whereby each amino acid is represented by a group with averaged electrostatic and van der Waals interactions. Finally, molecular dynamics (MD) was used to simulate 5 ns of multipeptides interacting with gold (Braun 2002). Also, MD-based simulations were used to find minimal energy orientations and unfolding trajectories of albumin subunits on graphite (Raffaini 2003).

Interface prediction relies on characteristics of residues found in interfaces of protein complexes. A number of databases of protein-protein interaction have been created, e.g http://protein3d.ncifcrf.gov/~keskino/,http://dockground.bioinformatics.ku.edu/,http://www.ces.clemson.edu/compbio/protcom/. The interfacial behaviour were identified by comparing interface and non interface portions of protein surfaces.

2.6 Examples of biomolecule characterization

The main goals of such studies would be to measure, predict and understand the protein conformation, surface coverage, superstructure and kinetic details of the protein–surface interaction. Proteins are often thought to denature at both solid– liquid and vapor–liquid interfaces, although they retain more structure on electrostatically neutral hydrophilic surfaces than on hydrophobic or charged surfaces. Kinetic measurements of adsorbed protein as a function of time and equilibrium adsorption isotherms have been measured using a variety of informative techniques, including optical waveguide lightmode spectroscopy (OWLS), ellipsometry and total internal reflectance fluorescence (TIRF). Studies have identified systematic effects of salt concentration, protein charge or dipole moment, and surface charge or hydrophobicity. Kinetic effects are often complex, in that long-time behaviour can differ from short-time behaviour, and final surface coverage can vary based on the rate at which protein was introduced into the system.

Behaviour is highly dependent on the individual nature of the protein and the surface involved. These complexities are often interpreted as arising from underlying structural phenomena (i.e. conformational change in the protein). For example, the orientation of fibronectin adsorbed on different polyelectrolytes was inferred from Optical Waveguide Lightmode Spectroscopy measurements. Similarly, the orientation and spreading behaviour of fibrinogen and lysozyme on hydrophobic and hydrophilic surfaces were inferred from kinetic measurements using Total Internal Reflectance Fluorescence (Gray 2004).

Direct observation of atomic-scale surface morphology is currently impossible, but progress has been made toward determining protein structure on a surface and useful structural information can be obtained. Biophysical methods for determining protein structure in solution (Circular Dicroism CD, IR spectroscopy, NMR and crystallography) are confronted with the challenge of the small heterogeneous samples present in a monolayer on a surface. Still, attenuated total reflection Fourier transform IR (ATR-FTIR) spectroscopy has been used to track the loss of secondary structure during insulin unfolding on a model lipid–water

interface (Sharp 2002), and as albumin or an antibody adsorbs to silica surfaces (Giacomelli 1999). Giacomelli and Norde (2001) have used CD together with probes of thermal stability to measure changes in the secondary structure of BSA before and after thermal denaturation. Long et al. (2001) used solid-state NMR techniques to determine the structure of the terminal helix of statherin on hydroxyapatite. This first high-resolution structural and dynamic characterization of a hydrated biomineralization protein adsorbed to its substrate is based on accurate measurements of distances between backbone carbonyl carbons and backbone nitrogens. Another study used NMR to isolate secondary structure changes in a model 13-residue peptide before and after adsorption to charged substrates (Read 2003). Biochemical techniques have also enabled researchers to infer much about themorphology of proteins on surfaces. Nanoscale imaging techniques have also been used. Atomic force microscopy (AFM) has been employed to study lysozyme (Kim 2002), ferritin (Johnson 2000) and insulin at surfaces, including the observation of surface denaturation (Sharp 2002). Scanning force techniques have been explored to study lysozyme and albumin topology and adhesion force (Mondon 2003), and scanning tunneling microscopy (STM) has also been used with lysozyme (Haggerty 1993(Gray 2004). Some methods are able to isolate information about particular regions of the protein. Hydrogen-exchange mass spectroscopy has been used to identify regions of lysozyme and α-lactalbumin that are accessible to solvent (i.e. partially unfolded) during chromatography under a range of solution conditions. An interesting conformational analysis was carried out on α-chimotripsin (Cht) during the interaction with a nanoparticle surface. The structure of (Cht) upon surface binding was monitored by both tryptophan fluorescence and circular dichroism (CD). By these techniques was possible to evidence the exposure of tryptophan residues to more polar environments as a consequence of protein denaturation and choose the best way of protein-surface interaction (Hong, 2004).

Many materials currently used in biomedical materials and tissue engineering scaffolds are modified directly with biomolecules, or the biomolecules can be conjugated through a hydrophilic layer [e.g., poly- (ethylene oxide)-based]. In addition to the biomaterials and tissue engineering fields, there is considerable interest in the patterned immobilization of active peptides and proteins, in affinity separations, diagnostics, proteomics, and cell culture technologies. Numerous studies have shown that peptide-modified surfaces influence cell attachment, shape, and function. For example, peptide coatings composed of the sequence arginine-glycine-aspartate (R-G-D) have been broadly used to promote cell adhesion to materials. A key element in optimizing the "biocompatibility" of peptide coatings is determining and controlling the secondary structure of the immobilized peptide. Solid-state

nuclear magnetic resonance (SSNMR) has the ability to probe structure and dynamics at specific residues in an immobilized peptide sequence. 13CSSNMR gives favorable signal if ~10^9 spins are present in the sample chamber. In the work carried out by Bower et al. (2005) a well structured peptide displaying a periodic alternation of leucine and lysine aminoacids: Ac-LKKLLKLLKKLLKL-NH2(LKR14) was synthesized. This peptide was shown in a previous study to assume a helical structure when adsorbed onto polystyrene beads After the synthesis the peptide was covalently tethered to gold nanoparticles coated with an alkanethiolate SAM and studied using a combination of SSNMR techniques. The aim of this work is to determine the influence that surface chemistry has on the structure and the activity of adsorbed peptide and protein coatings.

In the natural world, there are several examples of specific protein–surface interactions that can result in useful functions or materials. Biological examples have inspired the development of engineered systems that can take advantage of the functional properties of proteins to operate on the nanoscale. Sarikaya et al. (2003) have reviewed such molecular biomimetics, including a summary of 28 short peptide sequences that have been found to bind to solid surfaces ranging from platinum to zeolites to gallium arsenide. For materials synthesis, antibodies have been raised to bind to specific crystal surfaces to help control crystal growth (Sarikaya 2003, Geva 2003), and the growth of gold crystals has been controlled by peptide binding (Brown 2000). Brash and co-workers have explored adsorption onto liposomes (Price 2001), and have created lysine laden surfaces that can adsorb proteins that might dissolve clots (McClung 2003). Related ideas are explored in a recent review of the design of nanostructured biological materials through self-assembly (Zhang 2001). In another study, biotinylated peptide linkers were attached to a surface via streptavidin to bind fibronectin in an oriented manner (Klueh 2003). Such a material is then hoped to solicit a desired biological response (i.e. tissue integration). Perhaps the most exciting application is optical switching and modulation behaviour based on proteins affixed to a substrate (Ormos 2002). Clearly, there is great promise for careful control of the protein– surface interface to accomplish much, perhaps someday integrating electronics and nanotechnology with cell biology at the molecular level.

2.7 Conclusions

While a great deal has been learned from studies focusing on the amount and type of protein that interact with various types of surfaces, if membranes are to be designed to actually control protein interaction behaviour, it will be necessary to develop a much more detailed

understanding of the submolecular mechanisms involved in biomacromolecules-membrane interactions, and how this interactions influence the orientation, conformation, packing, and subsequent bioactivity of macromolecules.

References

Addadi L, Weiner S, Geva M: On how proteins interact with crystals and their effect on crystal formation. Z Kardiol 2001, 90(suppl 3):92-98

Agashe, M.; Raut, V.; Stuart, S.J.; Latour, R.A. Molecular simulation to characterize the adsorption behavior of a fibrinogen gamma-chain fragment. Langmuir 2005, 21, 1103–1117.

Andrew L. Zydney, Narahari S. Pujar, Protein transport through porous membranes: effects of colloidal interactions Colloids and Surfaces A: Physicochemical and Engineering Aspects, 138 (1998) 133-143

Balme S., Janot J., Déjardin P., Vasina E. N., Seta P., Potentialities of confocal fluorescence for investigating protein adsorption on mica and in ultrafiltration membranes, Journal of Membrane Science, 284, (2006) 198-204

Bowen W.R., Doneva T.A., Yin H.B., Atomic force microscopy studies of membrane—solute interactions (fouling)Desalination 146 (2002) 97-102

Bowen W.R., Hughes D.T., Properties of microfiltartion membranes. Adsorbition of bovine serum albumin at aluminium oxide membranes, J. Membr. Sci 51 (1990) 1898

Bower, P.V.; Louie, E.A.; Long, J.R.; Stayton, P.S.; Drobny, G.P. Solid-state NMR structural studies of peptides immobilized on gold nanoparticles. Langmuir 21 (2005) 3002–3007

Braun R, Sarikaya M, Schulten K: Genetically engineered goldbinding polypeptides: structure prediction and molecular dynamics. J Biomater Sci Polym Ed 2002, 13:747-757

Brown S, Sarikaya M, Johnson E: A genetic analysis of crystal growth. J Mol Biol 2000, 299:725-735

Chen S, Liu L, Zhou J, Jiang S: Controlling antibody orientation on charged self-assembled monolayers. Langmuir 2003, 19:2859-2864

Crespo J.P.S.G., Trotin M., Hough D., Howell J.A., Use of fluorescence labelling to monitor protein fractionation by ultrafiltration under controlled permeate flux, J. Membr. Sci. 155 (1999) 209

Cui Z., Protein separation using ultrafiltration –an exaple of multi-scale complex systems, China Particuology, 3 (2005) 343-348

Engel, M.F.M.; Visser, A.J.W.G.; van Mierlo, C.P.M. Conformation and orientation of a protein folding intermediate trapped by adsorption. Proc. Natl. Acad. Sci. U.S.A. 2004, 101, 11,316-321

Geva M, Frolow F, Eisenstein M, Addadi L: Antibody recognition of chiral surfaces. Enantiomorphous crystals of leucineleucine- tyrosine. J Am Chem Soc 2003, 125:696-704

Giacomelli CE, Bremer MGEG, Norde W: ATR-FTIR study of IgG adsorbed on different silica surfaces. J Colloid Interface Sci 1999, 220:13-23

Giacomelli CE, Norde W: The adsorption-desorption cycle. Reversibility of the BSA-silica system. J Colloid Interface Sci 2001, 233:234-240

Haggerty L, Lenhoff AM: Analysis of ordered arrays of adsorbed lysozyme by scanning tunneling microscopy. Biophys J 1993, 64: 886-895

Johnson CA, Yuan Y, Lenhoff AM: Adsorbed layers of ferritin at solid and fluid interfaces studied by atomic force microscopy. J Colloid Interface Sci 2000, 223:261-272

Keesom W.H., Zelenka r.L., Radke C.J., A zeta-potential model for surfactanat adsorbtion on an ionogenic hydrophobic surface, J.Colloid Interace Sci 125: 575 (1988)

Kim DT, Blanch HW, Radke CJ: Direct imaging of lysozyme adsorption onto mica by atomic force microscopy. Langmuir 2002, 18:5841-5850

Klueh U, Seery T, Castner DG, Bryers JD, Kreutzer DL: Binding and orientation of fibronectin to silanated glass surfaces using immobilized bacterial adhesin-related peptides. Biomaterials 2003, 24:3877-3884

Latour, R.A. Molecular modeling of biomaterial surfaces. Curr. Opin. Solid State Mater. Sci. 1999, 4, 413–417

Lautour R. A. , biomaterials: Protein-Surface interactions, Encyclopedia of biomaterials and biomedical engineering 2005, Taylor and Francis

Leos J. Zeman, Andrew l. zydney, mIcrofiltration and Ultrafiltration, priciples and application, New York, Basel 1996

Liu Z.M., Dubremez J., Richard V., Yang Q., Xu Z.K., Seta P., Useful method for the spatial localization determination of enzyme (peroxidase) distribution on microfiltration membrane, J. Membr. Sci. 267 (2005) 2

Long JR, Shaw WJ, Stayton PS, Drobny GP: Structure and dynamics of hydrated statherin on hydroxyapatite as determined by solid-state NMR. Biochemistry 2001, 40:15451-15455

Lundqvist, M.; Sethson, I.; Jonsson, B.-H. Protein adsorption onto silica nanoparticles: conformational changes depend on the particles' curvature and the protein stability. Langmuir 20 (2004) 10639–10647

Matthews, B.W. Studies on protein stability with T4 lysozyme. Adv. Prot. Chem. 1995, 46, 249–278

Mazzuca S., Giorno L., Spadafora A., Mazzei R., Drioli E.. Immunolocalization of _-glucosidase immobilized within polysulphone capillary membrane and evaluation of its activity in situ, Journal of Membrane Science 285 (2006) 152–158

McClung WG, Clapper DL, Anderson AB, Babcock DE, Brash JL: Interactions of fibrinolytic system proteins with lysine containing surfaces. J Biomed Mater Res 2003, 66A:795-801.
Mondon M, Berger S, Ziegler C: Scanning-force techniques to monitor time-dependent changes in topography and adhesion force of proteins on surfaces. Anal Bioanal Chem 2003, 375:849-855
Nystrom M. ; Lindstrom M. ; Matthiasson E., Streaming potential as a tool in the characterization of ultrafiltration membranes, Colloids and surfaces, 36 (1989) 297-312
Ormos P, Fabian L, Oroszi L, Wolff EK, Ramsden JJ, Der A: Protein-based integrated optical switching and modulation. Appl Phys Lett 2002, 80:4060-4062
Pontié, M.; Chasseray, X.; Lemordant, D.; Lainé, J.M., The streaming potential method for the characterization of ultrafiltration organic membranes and the control of cleaning treatments, Journal of Membrane Science, 129 (1997) 125-133
Price ME, Cornelius RM, Brash JL: Protein adsorption to polyethylene glycolmodified liposomesfromfibrinogensolution and from plasma. Biochim Biophys Acta 2001, 1512:191-205.
Raffaini G, Ganazzoli F: Simulation study of the interaction of some albumin subdomains with a flat graphite surface. Langmuir 2003, 19:3403-3412.
Ravihandran S, Madura JD, Talbot J: A Brownian dynamics study of the initial stages of hen egg-white lysozyme adsorption at a solid interface. J Phys Chem B 2001, 105:3610-3613.
Read MJ, Burkett SL: Asymmetric alpha-helicity loss within a peptide adsorbed onto charged colloidal substrates. J Colloid Interface Sci 2003, 261:255-263.
Ryosuke T. Masayuki N., Membrane charge of microporous glass membrane determined by the membrane potential method and its pore size dependency. Journal of Membrane Science,111 (1996) 19-26
Sarikaya M, Tamerler C, Jen AK, Schulten K, Baneyx F: Molecular biomimetics: nanotechnology through biology. Nat Mater 2003, 2:577-585.
Sharp JS, Forrest JA, Jones RA: Surface denaturation and amyloid fibril formation of insulin at model lipid-water interfaces. Biochemistry 2002, 41:15810-15819.
Shaw, W.J.; Long, J.R.; Campbell, A.A.; Stayton, P.S.; Drobny, G.P. A solid-state NMR study of dynamics in a hydrated salivary peptide adsorbed to hydroxyapatite. J. Am. Chem. Soc. 122 (2000) 7118–7119
Sheng Y-J, Tsao H-K, Zhou J, Jiang S: Orientation of a Y-shaped biomolecule adsorbed on a charged surface. Phys Rev E Stat Nonlin Soft Matter Phys 2002, 66:011911

Sousa H.A., Rodrigues C., Klein E., Afonso C.A.M., Crespo J.G., Immobilisation of pig liver esterase in hollow fibre membranes, Enzyme Microb. Technol. 29 (2001) 625
Sun, Y.; Latour, R.A. Prediction of the orientations of adsorbed protein using an empirical energy function with implicit solvation. Langmuir 2005, 21, 5616–5626
Tidwell, C.D.; Castner, D.G.; Golledge, S.L.; Ratner, B.D.; Meyer, K.; Hagenhoff, B.; Benninghoven, A. Static time-of-flight secondary ion mass spectrometry and x-ray photoelectron spectroscopy characterization of adsorbed albumin and fibronectin films. Surf. Interface Anal., 31 (2001) 724–733
Vermeer, A.W.P.; Norde, W. CD spectroscopy of proteins adsorbed at flat hydrophilic quartz and hydrophobic Teflon surfaces. J. Colloid Interface Sci. 225 (2000)394–397
Wagner, M.S.; Castner, D.G. Analysis of adsorbed proteins by static time-of-flight secondary ion mass spectrometry. Appl. Surf. Sci. 231–232 (2004) 366
Wagner, M.S.; Graham, D.J.; Ratner, B.D.; Castner, D.G. Maximizing information obtained from secondary ion mass spectra of organic thin films using multivariate analysis. Surf. Sci. 2004, 570, 78–97
Zhang S, Marini DM, Hwang W, Santoso S: Design of nanostructured biological materials through self-assembly of peptides and proteins. Curr Opin Chem Biol 2002, 6:865-871
Zhou J, Chen S, Jiang S: Orientation of adsorbed antibodies on charged surfaces by computer simulation based on a United-Residue Model. Langmuir 2003, 19:3472-3478

Chapter 3

Principles of Membrane Bioreactors and Membrane emulsification process

Introduction

In this chapter the basic principles that govern membrane bioreactors and in particular biocatalytic membrane reactors are reported.

In addition some highlights about membrane emulsification process are just recalled for clarity in the subsequent chapters.

3.1. Membrane (bio)reactors

Membrane (bio)reactors are combined processes in which it is simultaneously carried out a (bio)chemical conversion and a physical separation process.

The two main areas of membrane reactors are identified by the type of catalyst used, for example traditional chemical catalysts or catalysts of biological origin. The catalytic action of enzymes is extremely efficient and selective compared with chemical catalysts; the enzymes demonstrate higher reaction rates, milder reaction conditions and greater stereospecificity. The use of biocatalysts for large-scale production is an important application because it enables biotransformations to be integrated into productive reaction cycles.

The principal configuration both for chemical and biological reactor is where the membrane is a barrier that doesn't participate to the reaction, but allow a selective separation and when the membrane contains the (bio)catalyst and is catalytically active. In the first case the system was called membrane (bio)reactor, in the last case (bio)catalytic membrane reactor.

3.1.1 Membrane bioreactors

The name of bioreactors can be based on the type of solvent and/or separation process used and on the type of membrane used (e.g. flat-sheet membrane bioreactor) (Stratmann 2006).

Membrane bioreactors have several intrinsic advantages that make them a possible alternative system when compared with other, more conventional bioreactors. One of the main advantage is the possibility to use the biocatalyst in a continuous and thus intensive way, that contributes

in an increase of productivity and possibly to the economic viability of the process (Prazeres, 1994) (Chang 1991). Another important advantage is the continuous selective removal of the products from the reaction media.

Membrane bioreactor may display additional advantages in multiproduct systems. In such cases, if the membrane exhibit some selectivity toward the products, an enrichment of the product that is less rejected can be obtained in the outlet process stream. On the other hand, if a product is rejected by the membrane, it can be concentrated inside the system (Matson 1986).

In the case in which the membrane acts only as a separation unit, the biocatalyst can be continuously flushed along the membrane or segregated between the membrane module. In the first case the initial solution contains both the enzyme and the substrate, and the product is separated from the feed solution basing on its lower size (Fig 2.1 A). This type of reactor is based on the combination of a continuously stirred tank reactor (CSTR) with a separation unit (the membrane). This system is commonly useful for several type of reaction where a typical immobilized enzyme would not be effective. A particular case of this membrane reactor is the continuous membrane fermentator or cell-recycle membrane fermentator where it is necessary to separate the fermentation broth from the product stream (Prazeres 1994, Strathmann 2006).

In another configuration the catalyst is confined in a particular place in the membrane module space (Fig 3.1 B) and is not lost in the effluent stream, this system is called Membrane segregated enzyme reactor (MSER).

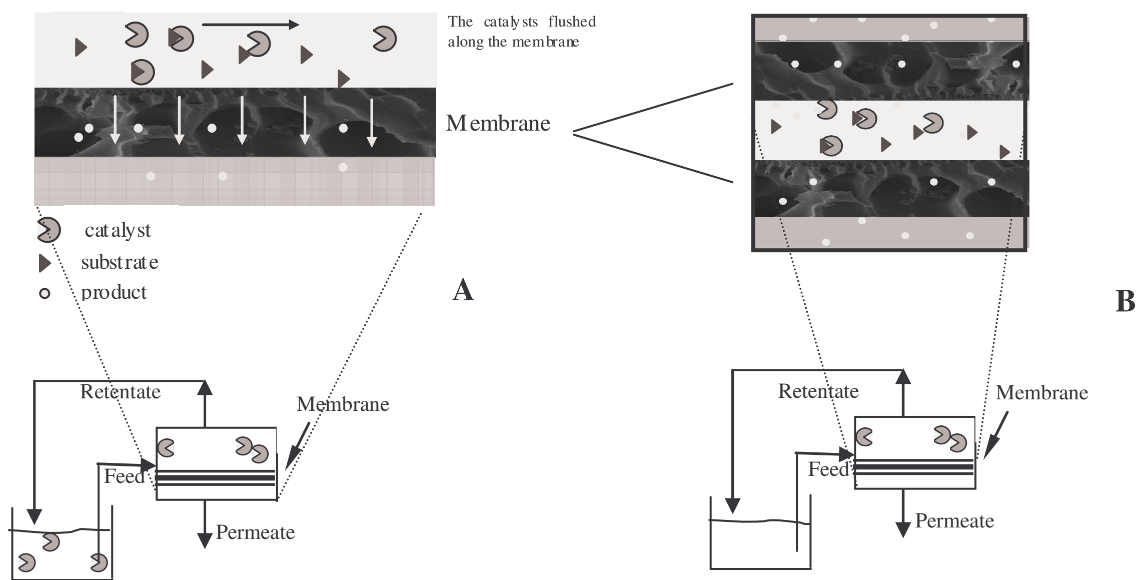


Fig.3.1 Schematic representation of membrane bioreactors in which membrane works as a separation unit. (A) UF membrane coupled with a Continuous Stirred Tank Reactor (CSTR), (B) Membrane segregated enzyme reactor (MSER).

The choice of reactor configuration depends on the properties of reaction system. For example, bioconversions for which homogeneous catalyst distribution is particularly important are optimally performed in a reactor with the biocatalyst compartmentalized by the membrane in the reaction vessel. The membrane is used to retain large components (the enzyme and the substrate, for example), while allowing small molecules to pass through (the product).

3.1.2.Biocatalytic membrane reactors

In this kind of reactor in addition to separation action membrane is also a catalytic unit. The different kind of biocatlytic membrane reactor configuration depends in which way the complex biocatalyst–loaded membrane is realized (Prazeres, 1994).

Immobilized biocatalyst have widespread applications in areas like organic synthesis, pollution control and for diagnostic purposes (Butterfield, 1996)(Amounas 2000, 2002). The selection of the membrane to be used in enzymatic membrane reactors should take into account the size of the (bio)catalyst (s), substrates (s), and products (s) as well as the chemical species of the species in solution and of the membrane itself. An important parameter to be used in this selection is the solute rejection coefficient, which should be zero for the product to facilitate permeation, and should be one for the enzyme to insure a complete retention of the catalyst inside the reaction system. The selectivity is normally associated with a discrimination based on size exclusion, but when a steric exclusion process may be present for molecule with size close to the pore size.

Immobilization eliminates the need to separate an enzyme from the product solution and allows these expensive compounds to be reused. In addition, the thermal stability, pH stability and storage stability of an enzyme may be increased as a result of immobilization.

The catalyst can be entrapped within the membrane, gelified on the membrane or bounded to the membrane (Strathmann, 2006). A scheme of immobilization techniques is reported in Fig. 3.2

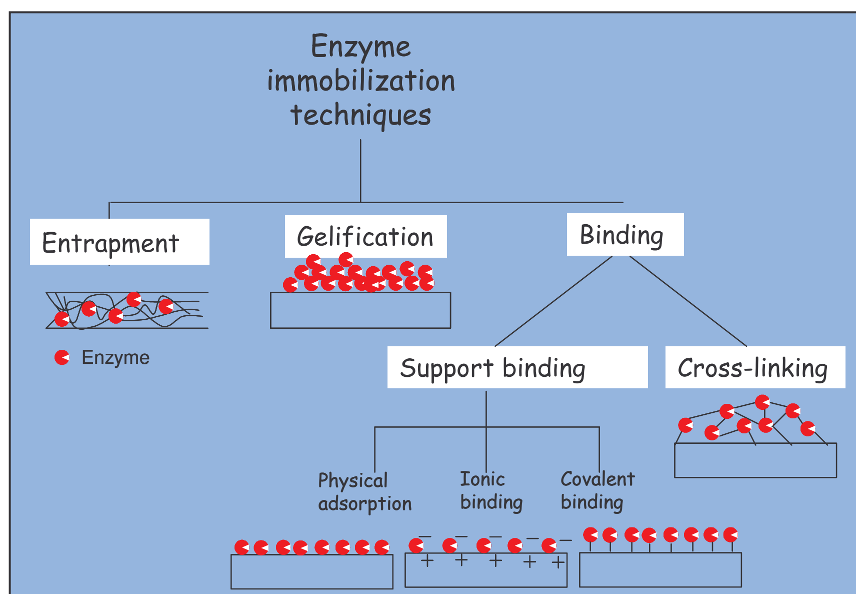


Fig. 3.2. Classification of enzyme immobilization techniques

3.1.2.1. Entrapment

The entrapment method of immobilization is based on the localization of an enzyme within the membrane. It is done in such a way as to retain protein while allowing penetration of substrate.

Asymmetric hollow fiber can provide an interesting support for enzyme immobilization. The enzyme can be entrapped in the sponge layer by cross-flow filtration in the case in which the pore size in the dense layer must retain the enzyme, permitting the passage of the substrate. The amount of biocatalyst loaded, its distribution and activity through the support and its lifetime are very important parameters to properly orientate the development of such systems. The amount of immobilized protein can be determined by the mass balance between initial and final solutions (Giorno, 1995).

A combined qualitative method (Mazzuca et al. 2006) merged from the classical *in situ* detection of enzyme activity and western blot analysis was applied for the first time to the capillary asymmetric polysulphone membrane reactor to determine simultaneously the enzyme (β-glucosidase) spatial distribution through the membrane thickness and along the membrane module and its activity after the immobilization through the observation in the light microscopy.

The transport of the substrate through the enzyme-loaded membrane is another important parameter. When the substrate passes through the membrane by convection the residence-time is an important factor to optimise (Strathmann, 2006).

3.1.2.2.Gelification

The gelification of the biocatalyst on the membrane is based on one of the main drawbacks of membrane processes: concentration polarization phenomena. Disadvantages of this systems is the reduction of the catalytic efficiency, due to mass transport limitations and the possibility to preferential pathways in the enzyme gel layer (Drioli 1989).

3.1.2.3.Binding to the membrane

The bound of the biocatalyst to the membrane can be divided in: physical absorption, ionic binding, covalent-linking and cross-linking.

3.1.2.4.Physical adsorption

Physical adsorption of an enzyme onto a solid is probably the simplest way of preparing immobilized enzymes. The method relies on non-specific physical interaction between the enzyme protein and the surface of the matrix.

A major advantage of adsorption as a general method of insolubilizing enzymes is that usually no reagents and only a minimum of activation steps are required. As a result, adsorption is cheap, easily carried out, and tends to be less disruptive to the enzymatic protein than chemical means of attachment, the binding being mainly by hydrogen bonds, multiple salt linkages, and Van der Waal's forces. In this respect, the method bears the greatest similarity to the situation found in biological membranes *in vivo* and has been used to model such systems. Because of the weak bonds involved, desorption of the protein resulting from changes in temperature, pH, ionic strength or even the more presence of substrate, is often observed. Another disadvantage is non-specific further adsorption of other proteins or other substances as the immobilized enzyme is used. This may alter the properties of the immobilized enzyme or, if the substance adsorbed is a substrate for the enzyme, the rate will probably decrease depending on the surface mobility of enzyme and substrate. (Goel, 1994).

3.1.2.5.Ionic binding and Covalent bond

The ionic binding consists in the bound of a charged enzyme to the support that has opposite charge.

The most intensely studied of the insolubilization techniques is the formation of covalent bonds between the enzyme and the support matrix. When trying to select the type of reaction by which a given protein should be insolubilized, the choice is limited by the fact that the

binding reaction must be performed under conditions that do not cause loss of enzymatic activity, and the active site of the enzyme must be unaffected by the reagents used.
The functional groups of proteins suitable for covalent binding under mild conditions include (i) the alpha amino groups of the chain and the epsilon amino groups of lysine and arginine, (ii) the alpha carboxyl group of the chain end and the beta and gamma carboxyl groups of aspartic and glutamic acids, (iii) the phenol ring of tyrosine, (iv) the thiol group of cysteine, (v) the hydroxyl groups of serine and threonine, (vi) the imidazile group of histidine, and (vii) the indole group of tryptophan. To prevent modification of enzymatic activity or complete inactivation of the immobilized protein it is important that the catalytic functional groups of the enzyme are not involved in the in the covalent linkage to the support. Unfortunately, many of the ractive groups suitable for immobilization are often situated in the active center of the enzyme. This problem can sometimes be eliminated by the immobilization in presence of the substrate (Jiang, 2000) or competitive inhibitor of the enzyme (Xie, 1999) (Charcosset 2006).

3.1.2.6.Cross-linking

Immobilization of enzymes can be also achieved by intermolecular cross-linking of the protein, either to other protein molecules or to functional groups on an insoluble support matrix. Cross-linking an enzyme to itself is both expensive and insufficient, as some of the protein material will inevitably be acting mainly as a support, resulting in relatively low enzymatic activity. Generally, cross-linking is best used in conjunction with one of the other methods.
Since the enzyme is covalently linked to the support matrix, very little desorption is likely using this method.

3.1.2.7. Site specific immobilization method

While immobilization of small molecule is typically easier, the active centre of larger proteins may no longer be accessible after immobilization. In these cases, improvement can be achieved by introducing a spacer molecule (Nouaimi 2001). Good steric accessibility of active sites can be obtained by oriented immobilization of glycoprotein enzymes through their carbohydrate moieties (Turkova 1999, Křenková 2004). Different approaches are developed in order to accommodate site-specific immobilization of enzymes with different structural characteristics, as gene fusion to incorporate a peptidic affinity tag at the N- or C- terminus of the enzyme; post translational modification to incorporate a single biotin moiety on enzymes; and site-directed mutagenesis to introduce unique cysteins to enzymes (Butterfield et al, 2001)

A small number of reactions have been designed to couple with functional groups on the protein other than the amino and phenolic residues. Aminoethyl cellulose has been coupled to the carboxylic acid residues of enzymatic protein in the presence of carbodiimide, and thiol residues of a protein have been oxidatively coupled to the thiol groups of a cross-linked copolymer of acrylamide and N-acryloyl-cystein.

It is possible in some cases to increase the number of reactive residues of an enzyme in order to increase the yield of insolubilized enzyme and to provide alternative reaction sites to those essential for enzymatic activity. As with cross-linking, covalent bonding should provide stable, insolubilized enzyme derivatives that do not leach enzyme into the surrounding solution. The wide variety of binding reactions, and insoluble carriers with functional groups capable of covalent coupling, or being activated to give such groups, makes this a generally applicable method of insolubilization, even if very little is known about the protein structure or active site of the enzyme to be coupled.

In literature there are various routes to carry enzyme immobilization creating a bound on supports, the principal strategies are based on chemical grafting or molecular recognition on porous supports. The techniques to understand the clustering of interaction between membrane and biomolecule is reported in chapter 6.

The sites involved in this chemistry: carboxylic acid, hydroxyls, amino or quaternary ammonium groups, are created on the surface of porous material by various means like the direct chemical surface treatment or the plasma or UV activation.

The reactive sites thus created allow the attachment of the enzyme by use of coupling reagents such as tosyl chloride, dicyclohexylcarbodiimide, glutaraldehyde etc.

Approaches aiming at creating bio-compatible environments consist in modifying the surface of polymeric membranes by attach of functional groups like sugars, polypeptides and then to adsorb the enzymes.

Another way considered as of bio-mimetic inspiration and which was shown to be efficient for enzyme attachment, it consists in using the very strong and specific interaction of the small protein avidin for the biotin (Amounas 2000,2002). The tetrameric structure of avidin permits itself to interact with four different molecules of biotin at the same time. Various proteins and enzyme could be easily biotinylated, and this mode of enzyme grafting has already been used for electrodes production as well as for membranes made up of conducting fibres.

3.1.3. Principles of biocatalytic membrane reactor

After the immobilization technique to characterize the bioreactor, in terms of catalytic aspects, different concepts have to be considered. Assuming complete mixing within the reactor so that enzyme and substrate concentration in the reaction vessel are uniform: if the enzyme is immobilized on the membrane surface, a decrease of substrate concentration in the retentate stream is observed, if the enzyme is immobilized within the pores, and not on the membrane surface, the concentration of the substrate in the retentate stream does not change, because only the solution that pass through the membrane is in contact with the biocatalyst. The substrate is then continuously recycled to the tank and fed to the enzyme-loaded membrane at a constant concentration.

In this last case the conversion degree was calculated as follows:

$$Conversion = \frac{C_r - C_p}{C_r}$$

Where C_r is the concentration of the substrate in the retentate solution and C_p in the permeate solution.. When the enzyme is present on the membrane surface and within the pores, it is necessary to take into account the conversion of the substrate in the retentate solution.

In this case the total conversion is calculated as:

$$Conversion = \frac{(C_r - C_p) + (C_i - C_r)}{C_i}$$

Where C_i is the initial substrate concentration.

Another important parameter for bioreactor characterization in terms of catalytic aspects is the calculation of the reaction rate.

By the total mass balance equation:

ACCUMULATION = IN-OUT+ PRODUCTION

$$\frac{dVC}{dt} = (FCi)_{IN} - (FCi)_{OUT} + (r_i V)_{system}$$

the reaction rate equation for a membrane reactor working at the steady-state was derived:

$$FC_f - FC_p + v_r V = 0$$

resolving for v_r the following is obtained:

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

In this equation v_r is the reaction rate (millimoles per cubic centimetre per minute), F is the permeate flow rate (cubic centimetre per minute), V is the reactor volume (cubic centimetre). For the various tank reactors systems the total mass balance equation is expressed in different form, basing on the fact that some terms in the equation are equal to zero. For batch system the flow component is zero for this reason the accumulation is equal to production (ACC=PROD), for semi-continuous reactor the flow term out is equal to zero (ACC=IN+PROD), for a Continuous Stirred Tank Reactor (CSTR) at the steady state the accumulation is equal to zero (IN-OUT+PROD=0) etc.

To calculate the reaction rate for a biological membrane reactor it is necessary to consider the the chemical equation that describes an enzymatic reaction that is:

The mathematical equation that describes the kinetic behaviour of enzyme is the Michaelis-Menten expression:

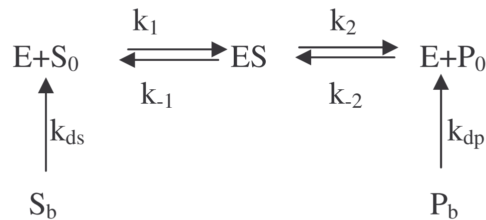
$$V_0 = \frac{V_{\max}[S]}{K_m + [S]}$$

V_o is the initial reaction rate, V_{max} the maximum reaction rate, K_m the Michaelis-Menten constant and [S] the substrate concentration. Rearranging the M-M equation in linear form the Lineweaver-Burk equation is obtained. This is a graphical method to directly estimate K_m and V_{max}:

$$\frac{1}{V_0} = \frac{K_m}{V_{\max}} \frac{1}{S} + \frac{1}{V_{\max}}$$

If $\frac{1}{V_0} vs \frac{1}{[S]}$ data are plotted, a straight line is obtained, where the intercept to the y axis is $\frac{1}{V_{\max}}$ and the slope is $\frac{K_m}{V_{\max}}$.

The kinetic reaction for an immobilized enzyme is usually represented as follow:



S_0 and P_0 are the concentration of the substrate and product near the immobilized enzyme, Sb and Pb are the concentration of substrate and product in the bulk phase. The constants k_{ds} k_{dp}, are related to the diffusion of substrate from the bulk to the enzyme and of the product from the enzyme to bulk phase, these constants take into account diffusion phenomena near the enzyme-membrane wall. In fact the substrate and the product have to different and opposite concentration gradient through the immobilized enzyme matrix. The concentration of the substrate in the bulk phase decrease near the support due to diffusion phenomena continuing its decrease near the enzyme matrix due to diffusion effect and reaction. An opposite behaviour of concentration gradient is observed for the product, near the enzyme matrix the product concentration is high due to reaction, far from the support it decreases arriving at a steady state in the bulk solution.

3.1.3.1 Enzyme immobilized on the surface

For the enzyme immobilized on the surface, a stationary film also called Nerst diffusion layer, is formed, attached to the enzyme-membrane surface. This layer limits the diffusion of the substrate and for this reason the concentration of the substrate in the bulk solution decreases near the immobilized enzyme matrix.

At the steady state, at the interface, the mass transfer of the substrate is balanced from the reaction and consequently from the substrate consumption. In this case the Michaelis-Menten equation take into account the different substrate concentration in the bulk solution and near the surface:

$$J_s = k_s (S_0 - S) = \frac{V_{max}[S]}{K_m + [S]}$$

In this equation S and S_0 are the substrate concentration in the bulk and at the immobilized enzyme interface respectively and k_s is the mass transfer coefficient.

The ratio between the maximum reaction rate and maximum mass transfer rate is given by the Damköhler number:

$$Da = \frac{V_{max}}{k_s S_0}$$

If Da <<1 the transfer rate is larger than the reaction rate, and this means that the system works at a low mass transfer resistance. This is the case known as reaction limited regime. In this system the following equation:

$$V_{kin} = \frac{V_{max}[S]_b}{K_m + [S]_b}$$

can be assumed.

If Da >> 1, the reaction rate is larger than the mass transfer, this is the case known as diffusion-limited regime and $V_{diff} = k_s [S]_b$.

The Damköhler number is also the ration between V_{max}/K_m to k_s , V_{max}/K_m is also the slope of $1/V_0$ versus 1/ K_m and k_s is the slope of V_{diff} versus $S_{b.}$

The mass transfer influence on the reaction is represented from the factor η:

$$\eta = \frac{Observed\ reactio\ rate}{Rate\ observed\ without\ mass\ trasfer\ resis\tan ce}$$

If η is ≤ 1 the mass transfer resistance is high and this cause a reduction of the observed activity of the catalyst. The relationship between Da and η is that when Da approaching zero η approaches 1.

3.1.3.2. Enzyme immobilized into the porous matrix

To calculate the observed substrate conversion through an enzyme-loaded-support immobilized into the internal surface, it is necessary take into account the concentration profile within the diffusion layer.

Beside substrate diffusivity in the bulk phase, the diffusion rate through a porous support is influenced by several factors. The effective diffusion coefficient is described by:

$$D_{eff} = D_{S_0} \frac{\varepsilon_p}{\tau} \frac{K_p}{K_r}$$

ε_p is the porosity (or area of support/ area of pores); τ is the tortuosity (the geometry of the pore is not tubular, diffusion occur changing direction continuously). The tortuosity factor can assume a value in a range of 1.4 to 7; K_p/K_r is the restricted diffusion roughly estimated as $[1-r_{substrate}/r_{pore}]^4$, that takes into account the dimension of the pore in relation with the dimension of the substrate, that can have similar dimension and lead to a situation of restricted diffusion.

The influence of diffusion within porous catalysts upon reaction kinetic was studied in 1930 (Bailey 1986), this effect was studied on a planar membrane with an immobilized enzyme uniformly distributed. Combining the steady-state diffusion equation with the applicable kinetics rate expression gives:

$$D_{eff} \frac{d^2[S]}{dx^2} - \frac{V_{max}[S]}{K_m + [S]} = 0$$

D_{eff} is the effective diffusivity, this means that at the steady state the substrate diffusion rate, through a porous matrix, is equal to the rate conversion.

Also in an immobilized system is possible to evaluate if the reaction is limited by kinetics or mass transport by the Thiele modulus ø, given by:

$$\phi = L \left(\frac{V_{max}}{D_{eff} K_m} \right)^{1/2}$$

which has the meaning of a reaction rate/diffusion rate.

3.2. Highlights of Membrane emulsification

In this paragraph a general overview about membrane emulsification process is given. These informations are necessary to understand the integration of membrane emulsification process and biocatalytic membrane reactor described in chapter 8.

Membrane emulsification is a relatively new technology in which membranes are not used as selective barriers to separate substances but as microstructures to form droplets with regular dimensions, i.e. uniform or controlled droplets size distribution. Membrane emulsifications can be generally distinguished in: (i) direct membrane emulsification (DME), in which the disperse phase is directly fed through the membrane pores to obtain the droplets. and (ii) pre-mix membrane emulsification, in which a coarse pre-mixed emulsion is pressed through the membrane pores to reduce and to control the droplet sizes.

In general, in the direct membrane emulsification, the disperse phase is pressed through a microporous membrane and droplets are formed at the opening of the pore on the other side of the membrane, which in contact with the continuous phase. Here, droplets reached a critical dimension can detach either for spontaneous deformation or sheared by the continuous phase flowing parallel to the surface. In the former case, the driving force for the droplet formation is the surface free energy minimization, that is, the droplet is formed by spontaneous deformation tending to form a sphere. For example, in quiescent conditions the droplets are formed by means of this mechanism. In the latter case, the shearing stress generated by the continuous phase is the driving force of the droplet detachment. For example, in the cross-flow membrane emulsification (CDME) and stirred membrane emulsification droplets are formed by this mechanism (Fig 3.3).

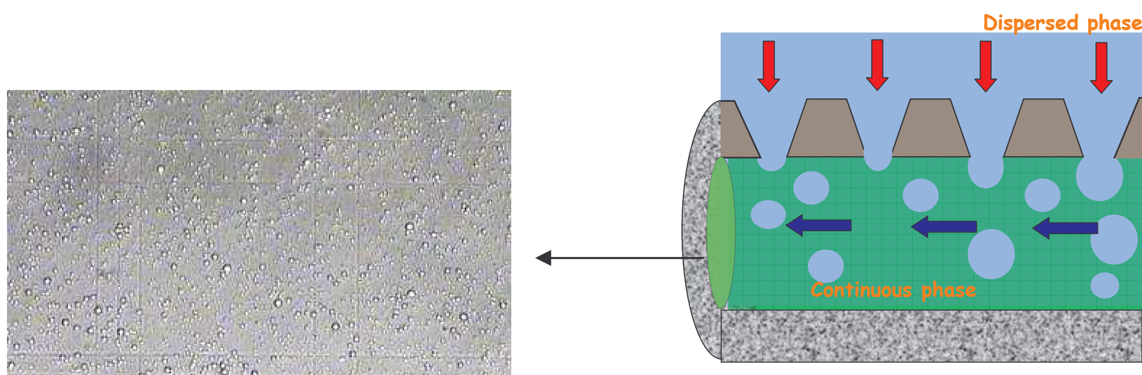


Fig. 3.3. Scheme of principle of cross flow membrane emulsification process in tubular membrane and emulsion image observed by optical microscopy.

Both direct and pre-mix emulsification can be obtained with a continuous phase flowing along the membrane surface (i.e. cross-flow, stirring). However, it is important to distinguish between the droplet formation mechanism and the macroscopic operation procedure. In other terms, often, in the literature, the "cross-flow" term is used to indicate that the continuous phase is flowing along the surface, but this does not guarantee that the shear stress is the driving force for the droplet detachment, as long as the appropriate conditions are not verified.
A peculiar advantage of membrane emulsification is that both droplet sizes and size distributions may be carefully and easily controlled by choosing suitable membranes and focusing on some fundamental process parameters reported below. Membrane emulsification is also an efficient process, since the energy density requirement (energy input per cubic meter of emulsion produced, in the range of 10^4-10^6 Jm^{-3}) is low with respect to other conventional mechanical methods (10^6-10^8 Jm^{-3}), especially for emulsions with droplet diameter smaller than 1 μm. The lower energy density requirement also improves the quality and functionality of labile emulsion ingredients, such as bioactive molecules. In fact, in conventional emulsification methods, the high shear rates and the resulting increase of the process temperature have negative effects on shear- or temperature-sensitive components. The shear stresses calculated for a membrane system are much less and it is possible to process shear sensitive ingredients.
The droplet size, its dispersion and the droplet formation time depend on several parameters: (i) *membrane parameters*, as pore size distribution, pore border morphology, number of active pores, porosity, wetting property of the membrane surface, (ii) *operating parameters*, as cross-flow velocity (i.e. wall shear stress), transmembrane pressure and disperse phase flow, temperature, as well as the membrane module used (tubular, flat, spiral-wound);and (iii) *phase parameters*, as dynamic interfacial tension, viscosity and density of processed phases, emulsifier types and concentration. Droplets size distribution and disperse phase percentage determine the emulsion properties characterizing the final formulation for an intended use (Giorno et al. 2009).

References

Amounas M., Innocent C., Cosnier S., Seta P.. J. Memb. Sci. 2000,176 169-176.

Amounas M., Magne V., Innocent C., Dejean E., Seta P.. Enzyme and Microbial Technology 2002, 31, 171-178.

Bailey J.E., Ollis D.F., Biochemical engineering fundamentals, New York: McGraw-Hill 1986, 97

Butterfield D.A., Biofunctional membranes, Plenum Press NY 1996, 117-129.

Butterfield D.A., D. Bhattacharyya, S. Daunert, L. Bachas, Journal of Membrane Science, 2001, 29-37

Chang H. N. , Furusaki S., Advances in Biochemical Engineering/Biotechnology, Springer Berlin Heidelberg, 1991, 44, 27-64

Charcosset C., Biotechnology Advances 2006, 24, 482-492

Drioli E., Iorio G., Catalano G., Handbook of industrial membrane technology. New York: Noyes Publications 1989, 401-481

Giorno L., De Luca G., Figoli A, Piacentini E., Drioli E., in Membranes Operations,. Innovative separations and transformations, Eds. E. Drioli and L. Giorno, Wiley-VCH, (2009) in Press

Giorno L., Molinari R., Drioli E., Bianchi D., Cesti P., J.Chem. Tech. Biotechnol. 1995, 64, 345

Goel M.K, www.rpi.edu/dept/chem-eng/Biotech-Environ/IMMOB/goel2nd.htm, 1994

Jiang H., Zou H., Wang H., Ni J., Zhang Q., Zhang Y.. J Chromatogr A. 2000, 903,77-84.

Matson L., Quinn J.A., Ann. NY Acad Sci 1986, 469, 152-165.

Mazzuca S., Giorno L., Spadafora A., Mazzei R., Drioli E., Journal of Membrane Science 2006, 285, 152–158

Nouaimi M., Möschel K., Bisswanger H., Enzyme Microb. Technol. 2001, 29 567-574

Prazeres D.M.F., Cabral J.M.S., Enzyme and Microbial Technology 1994,16, 738-750

Strathmann H., Giorno L., Drioli E., An introduction to Membrane Science and Technology, Rome 2006

Turková J., J Chromatogr B Biomed Sci Appl. 1999, 722, 11-31S.

Křenková J., Foret F., Electrophoresis 2004, 25, 3550.

Xie S., Svec F., J.M.J. Fréchet, Biotech. Bioeng. 1999, 62 , 30.

Chapter 4

Biochemical Membrane reactors in industrial process and in patent development

4.1. Biochemical Membrane Reactors in industrial process and in patent development

4.1.1. Introduction

Biochemical membrane reactors are systems able to optimally integrate and intensify chemical transformations and transport phenomena in a single unit. The transformation is promoted by a catalyst of biological origin (commonly named biocatalyst) while the transport is governed by a membrane operation (i.e. by a driving force acting through a micro- nano-structured porous or dense membrane). Transport can be appropriately tuned so that to control reagent supply to the catalyst and/or product removal from the reaction site.

The fundamentals of biochemical membrane reactors are reported in a previous chapter. Here some highlights are just recalled for clarity in the subsequent discussion.

The applications presented refer to both main reactor configurations, i.e. the configuration in which the membrane does not contribute to the reaction but only controls mass transport and the configuration in which the reaction also occurs at the membrane level.

The present work will mainly focus on biochemical membrane reactors operated at production scale give an overview of systems of potential interest studied at laboratory level.

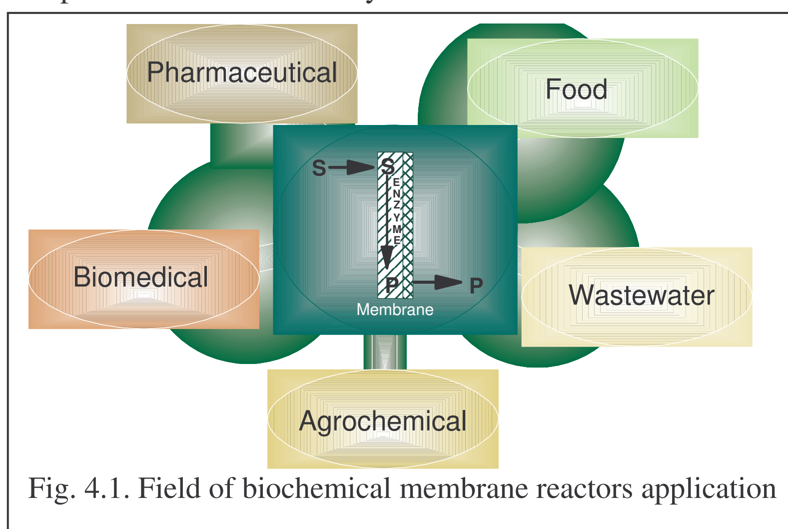


Fig. 4.1. Field of biochemical membrane reactors application

Despite the various field of applications (Fig. 4.1.), in this work industrial sectors such as pharmaceutical, food and biotechnology will be considered. Waste water treatment and biomedical

applications are discussed in other chapters.

The catalytic action of biocatalysts (enzymes, abzymes, antibodies, cells) is extremely efficient and selective compared to conventional chemical catalysts. They demonstrate higher reaction rates, milder reaction conditions and greater stereospecificity. Most of these properties come from the high molecular flexibility biocatalysts exhibit. On the other hand, this is also the origin of their major limit that brakes their application at large scale, i.e. the molecular stability, and then the catalyst life time.

The use of biocatalysts in combination with membrane operations permits to overcome drawbacks enabling biotransformation to be integrated into continuous production lines. These systems being able to work at time invariant conditions at steady state permit a better control of reaction conditions with an increase of life time, productivity and economic viability of the process. In addition, the separation, purification and concentration of the obtained product can occur in a single integrated unit operation. Thanks to the biocatalyst and membrane selectivity the mass intensity can be very high, with no by-products formation while producing high added value co-products.

4.1.2. Applications at industrial level

Despite their great advantages, the application of biochemical membrane reactors at industrial scale in pharmaceutical, food and biotechnology is still limited. Major reasons for this include the non adequate research efforts devoted to the field, lack of predictive and holistic approach. A clear example of this situation is constituted by the commercial success submerged membrane bioreactors met in waste water treatments. In this field, the technology was pushed by research efforts promoted to face lack of clean water and to meet regulations about waste water discharge in the environment. More stringent regulations about eco-compatibility of industrial processes will necessarily promote technological advances also in other industrial sectors. For example, considering the mass of wastes compared to the mass of product, it appears that pharmaceutical industry used less advanced technology than oil refinery (Fig. 4.2. a). Due to the orders of magnitude difference between the two sectors in terms of tons of productions (Fig. 4.2. b), the impact of pharmaceutical industry is of course much lower, but it is evident that in this field there is a much higher potential for knowledge-based technologies.

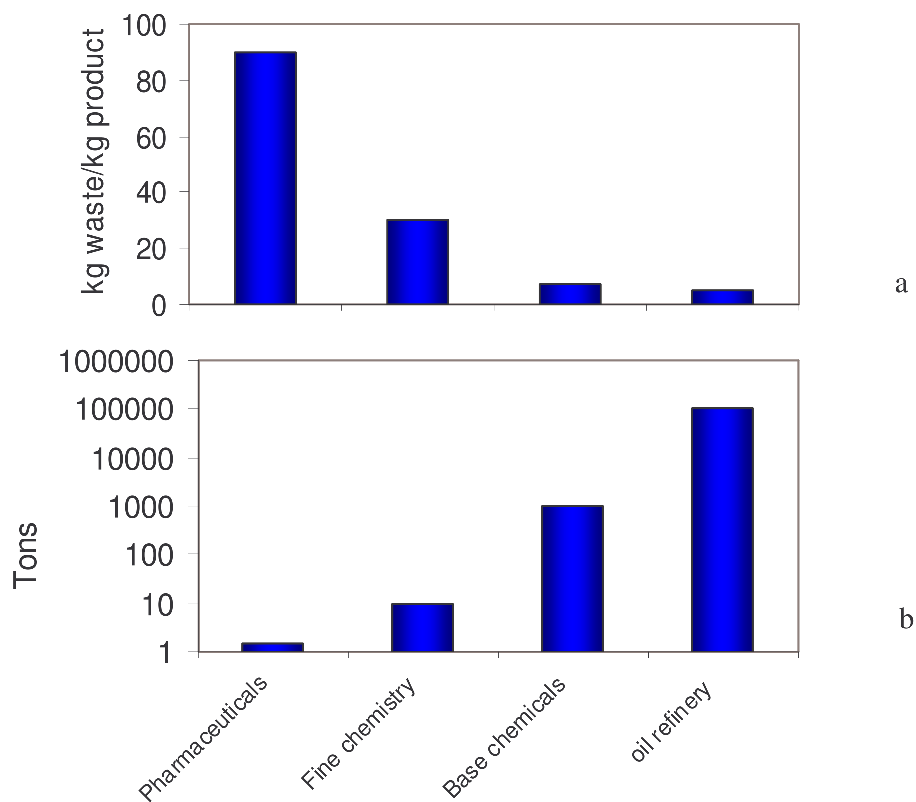


Fig. 4.2. Wastes produced related to mass of product in industries (1)

Table 4.1. summarizes the most common examples of biochemical membrane reactors patented and whose robustness has been proved at industrial production scale. Table 4.1 illustrates the type of application, the biocatalyst used and the way it is used in the membrane reactor. When the enzyme is used as free, the membrane serves to separate the reaction product, whilst when it is immobilized the membrane hosts both reaction and separation.

Table 4.1. Biochemical membrane reactors in industrial processes

Biocatalyst	Status	Application
Lactase	immobilized	Hydrolysis of beta-D-galactosidic linkage of lactose milk (Industrial scale)(2)
Glucose isomerase	immobilized	Conversion of D-glucose to D-fructose (Industrial scale) (3)
Penicillin acylase	free	Production of antibiotics (Industrial scale) J. (4)
Acylase	immobilized	Production of L-aminoacids (Industrial scale)(5)
E. Coli	immobilized	Production of L-aspartic acid (Industrial scale) (6)
Pseudomonas dacunahe	immobilized	Production of L-alanine (Industrial scale)(7)
Aminoacilase, and dehidrogenase	Freeb and immobilized	Production of L-aminoacids (8)(9)(10)
Brevibacterium ammoniagenes	immobilized	Production of L-malic acid (Industrial scale) (11)
Pectic enzymes	free or immobilized	Hydrolysis of pectins to improve processability (industrial scale) (12)(13)
Thermolysin	Immobilized	Production of aspartame (Industrial scale) (14)
Lipase OF 360	immobilized	Production of diltiazem chiral intermediate (industrial scale) (15)(16)
Trypsin	free	Production of casein bioactive peptide (patented) (17)(18).
Protease	immobilized	Hydrolisis of caroteno-proteins (patented)(19)
Acetyl transferase from Taxus	immobilized	Production of baccatin III (patented) (20)
Lipase	free	Production of fatty acid (patented) (21)
Cells	-	Linear or membrane-like biodevices and a bioreactor in which adhesive cells are anchored at high density (patented) (22)
Pancreatic cells	immobilized	Artificial organs and implantable bioreactors (patented) (23)(24)
Cells	free	Continuous cell culture (patented) (25)
Stem cells	-	Cell expansion apparatus (patented) (26)
Viruses, virus particles, antibodies and proteins	free	Production of a concentrated solution from biological substances (patented)(27)
Cells		Delivery of drugs or genes to individual cells (patented) (28)

4.1.2.1 Pharmaceutical applications

The use of Membrane Bioreactors in pharmaceutical field have been documented for the production of amino acids, antibiotics, anti-inflammatories, anticancer drugs, vitamins, optically pure enantiomers.(10)(29)(30)(31).

Examples at industrial scale of membrane bioreactors in pharmaceutical field include the production of amino acids with simultaneous regeneration of NADH which has been commercialized by Degussa Company in Germany (10). In Japan the Kao Corporation investigated the so called sandwich-reactor for hydrolysing triglycerides (32), Nitto Electric Industries immobilized cyclomaltodextrin glucanotransferasi on an hollow fiber membrane and investigated production of cyclodestrins from starch (32).

Membrane bioreactors have been reported for the production of diltiazem chiral intermediate with a multiphase/extractive enzyme membrane reactor (15)(16). The reaction was carried out in a two-separate phase reactor. Here the membrane had the double role of confining the enzyme and keeping the two phases in contact while maintaining them in two different compartments. This is the case of the multiphase/extractive membrane reactor developed on productive scale for the production of a chiral intermediate of diltiazem ((2R,3S)-methylmethoxyphenylglycidate), a drug used in the treatment of hypertension and angina (15). The principle is illustrated in Fig 4.3, the reactant was fed in solvent while the product was extracted in water. The lipase was immobilized by entrapment method on asymmetric PAN hollow fiber membranes. The process was run for several years with module for the production plant of 60 m^2 of active membrane area.

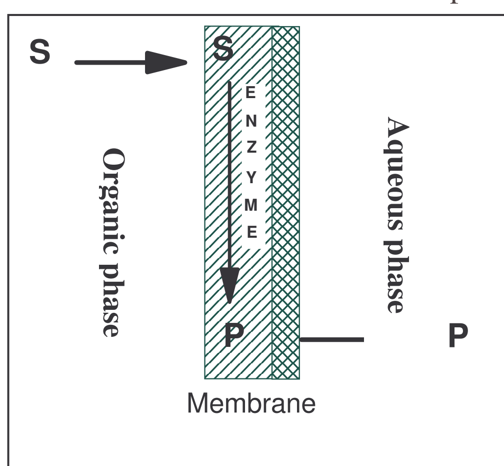


Fig 4.3. Schematic representation of multiphase membrane reactor

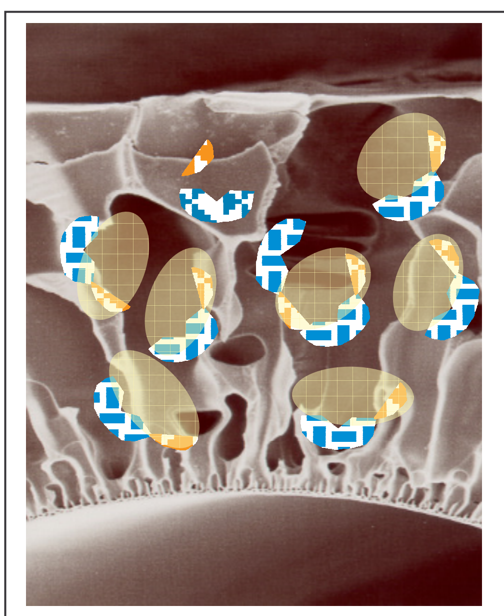


Fig 4.4. Representation of emulsion enzyme membrane reactor

A further improvement of multiphase reactor concept using lipase for enantioselective transformation has been recently reported, i.e. an emulsion enzyme membrane reactor. Here, the

organic/water interface within the pores at the enzyme level is achieved by stable oil-in-water emulsion, prepared by membrane emulsification. In this way, each pore forms a microreactor containing immobilized enzyme (Fig.4.4) (33). In the membrane pores, the enzyme is able to work in the same conditions as in the stirred tank reactor, but with no shear stress due to stirring.

This configuration improved the selectivity and productivity of the biocatalytic system as well as its catalytic stability, confirming that the observed inversion relationship between activity and stability of immobilized enzyme is not a general rule.

Other biochemical membrane reactors applications include the synthesis of lovastatin with immobilized *Candida rugosa* lipase on a nylon support (34); the synthesis of isomalto oligosaccharides and oligodextrans in a recycle membrane bioreactor by the combined use of dextransucrase and dextranase (35), the production of a derivative of kyotorphin (analgesic) in solvent media using α-chymotrypsin as catalyst and α- alumina mesoporous tubular support (36), and biodegradation of high-strength phenol solutions by *Pseudomonas putida* using microporous hollow fibers (37).

A particular application of membrane bioreactors, patented in 2005 (20), concerns the production of an anti tumor substance (paclitaxel). Since a full synthesis of paclitaxel is not possible due to its low yield, a semi synthesis of 10-deacetyl-baccatin III (10-DAB) was carried out from which baccatin III was produced in an enzyme reactor. The enzyme reactor comprised a hollow fiber polymeric ultrafiltration membrane, with immobilized acetyl transferase from *Taxus species.* The process enabled the production of baccatin III without requiring complicated purification steps of the acetyl transferase. The purification of the baccatin III is also distinctly made easier (20)

Membrane bioreactors can be easily integrated with other systems for example with delivery of drugs or genes to individual cells achieved on a nanoscale using electroporation techniques. In one method developed in a recent patent, a flow-through bioreactor having an inlet and an outlet connected by a flow chamber and a nanoporous membrane positioned in the flow chamber was used (28).

Recent works in pharmaceutical field using MBR technology are related to optical resolution of racemic mixtures or esters synthesis. The kinetic resolution of (*R*,*S*)-naproxen methyl esters to produce (*S*)-naproxen in emulsion enzyme membrane reactors (E-EMRs) where emulsion is produced by cross-flow membrane emulsification (38)(39), and of racemic ibuprofen ester (40) were developed. The esters synthesis like for example butyl laurate, by a covalent attachment of *Candida antarctica* lipase B (CALB) onto a ceramic support previously coated

by polymers was recently described (41). An enzymatic membrane reactor based on the immobilization of lipase on a ceramic support was used to perform interesterification between castor oil triglycerides and methyl oleate, reducing the viscosity of the substrate by injecting supercritical CO_2 (42).

The production of aromatic compounds by membrane bioreactor is widely studied and some examples also patented. Aromatic compounds are important substances in pharmaceutical, food, and cosmetic industry due to its natural properties and because they are strong antioxidant molecules with a strong free radical scavenging activity.

The hydrolysis of caroteno-proteins for the production of astaxanthin using protease was developed in an enzymatic membrane bioreactor (19), in which it was simultaneously carried out the concentration of the protein fraction by ultra-filtration and the separation of the pigments in the permeate.

Terpene esters belong to a great family of aromatic compounds, are important flavoring and widely used in pharmaceutical and food industry. An important terperne ester is α-pinene oxide, its biotransformation to isonovalal using resting cells of *Pseudomonas fluorescens* NCIMB 11671 was evaluated in a membrane bioreactor (43). Production of geranyl acetate, one of the most known aromatic compound, was studied using lipase CAL-B immobilized on polymer membranes by sorption and chemical binding (44).

4.1.2.2. Food applications

The main applications of biocatalytic membrane reactors in the food sector include: reduction of the viscosity of juices by hydrolysing pectins, reduction of the lactose content in milk and whey by its conversion into digestible sugar, treatment of musts and wine by the conversion of polyphenolic compounds and antocyanes and the removal of peroxides from diary products.

The interactions between pectins and sugars (rhamnose, arabinose and galatose) are principal responsible for the high turbidity and viscosity of fruit juice. Pectinases immobilized in membranes are used to reduce the viscosity of fruit juice (12)(13).

One of the first cases of the application of membrane bioreactors in food processes was the production of milk with low lactose content. β-galactosidase was entrapped into cellulose fibres acetate to carry out the hydrolysis of milk and whey lactose (2) recently the system was improved by the use of microfiltration and by UV irradiation of the enzyme solution to avoid growth of microorganism (45).

The use of membrane reactors as continuous systems for the hydrolysis of lactose (present in whole milk or cheese whey) is an effective technique running at large scale. Intolerance to milk is not only caused by lactose, but also by high molecular weight proteins. In fact, some children and old people have difficulty to hydrolyse proteins with molecular weight higher than 5 kDa. In other words, they cannot digest such proteins, which induce stomach ache and can also lead to allergy. The hydrolysis of high molecular weight proteins into polypeptides lower than 5 kDa in biocatalytic membrane reactors is a new approach to produce low allergenic fresh milk with improved properties compared to the reconstituted powder milk currently used. The biocatalytic membrane reactor can be designed so that the hydrolysed fragments equal or lower than 5 kDa can be removed through membrane of appropriate cut-off while retaining the non-hydrolysed proteins. In order to achieve high efficiency, the hydrolytic step should be part of an integrated system where up- and down-stream of milk is properly considered. Biocatalytic membrane reactors can also be used to valorise co-products of cheese-making processes. In fact, it is possible to increase the cost effectiveness of cheese-making processes and reduce waste simply by recovering and reusing compounds present in waste streams. The whey proteins (such as α-lactalbumin), which have excellent functional properties, can be recovered by ultrafiltration and hydrolysed to produce many useful pharmaceutical intermediates. In addition, permeates from the ultrafiltered milk and whey contain lactose, which can be recovered and used in the production of glucose and galactose syrup.

Other important application in food industry running at large scale are the production of L-aspartic acid with *Escherichia coli* entrapped in polyacrilamides (6), the immobilization of thermolysin for the production of aspartame (14), The production of L-alanine by Tanabe Seiyaku (7), the production of fructose concencentrated syrup (3), the production of L-malic acid by the use of *Brevibacterium ammoniagenens* immobilized in polyacrilamide by entrapment immobilization methods (Takata), and L-aminoacids production by immobilized aminoacylase (5).

Biocatalytic membrane reactors are also used for the treatment of musts and wines by the conversion of polyphenolic compounds and anthocyanes. Laccase is used to oxidize polyphenols in solution and anthocianase is used immobilized on synthetic and natural polymers to hydrolyse anthocyanes.

During the maturation process, a secondary fermentation occurs that converts malic acid into lactic acid. Control of this reaction will enable the production of a product with good organoleptic properties. In comparison with batch fermentation, membrane bioreactors

achieve remarkably elevated cell concentrations and productivity. In this type of bioreactor, a membrane has been installed to prevent washout of yeast when fermented stream is withdrawn from the reactor. This apparatus makes it possible to keep the cell concentration in the reactor high while reducing product inhibition by replacing product-containing broth with fresh medium.

Recent patented works were also reported related to the design of membrane bioreactors.

A membrane biological reactor of flat x-plate system comprising a thermostat, and a free system comprising hydrophilic membrane with cut-off value of 30100 kDa at 15-65[deg]C using lipase enzyme was developed and patented for the production of fatty acids by enzymatic hydrolysis of vegetable or animal oils or fats (21). Membrane bioreactors were also used to improve existing systems for different applications, like cells nutrition and growth, and as delivery systems for genes and drugs (22)(23)(24)(25)(28), to produce concentrated solution consisting of biological substances such as, e.g. viruses, virus particles, antibodies and proteins (27)

The design of food products that confer a health benefit is a relatively new trend, and recognizes the growing acceptance of the role of diet in disease prevention, treatment and well-being. This change in attitude for product design and development has forced organizations and industries involved in formulating foods for health benefit into new areas of knowledge.

Recent works in literature were devoted to improve the production of food similar to existing one that is less dangerous in compromised diet for the production of functional food or the production of nutraceuticals.

Palatinose (isomaltulose, 6-O-a-D-glucopyranosyl-D-fructose) a natural substance whit sweetening power of about 45% less of that of sucrose, and xilitol, where insulin is not involved in its methabolism, can be used as sugar substitutes in diabetic subjects. Recently it was produced a complete conversion of concentrated sucrose solutions into palatinose immobilizing in a hollow-fibre membrane reactor *Serratia plymuthica* cells (46). *Candida tropicalis*, an osmophilic strain isolated from honeycomb, was used to produce xilitol recycled in a submerged membrane bioreactor with suction pressure and air sparging, obtaining the highest productivity of xilitol 12.0 g l^{-1} h^{-1} (47).

Octenylscuccinate derivatives of starch are attracting growing attention of food technologist as potential additives as emulsifying agents. The results obtained using the technology of membrane bioreactors indicated that the hydrolysis of sodium octenylsuccinate starch derivatives leads to products which reveal the surface activity, irrespective of the type as well

as the amount of enzyme used in hydrolysis process. The application of the UF membrane bioreactor to enzymatic hydrolysis could be the way of intensification of the production process (48).

A very interesting field in membrane bioreactors is the production of cyclodextrins or oligosaccharides. In general, they have applications in food pharmaceutical, cosmetic agricultural and plastic industry as emulsifiers, antioxidant and stabilizing agents. In food industry cyclodextrins are employed for the preparation of cholesterol free products. The use of enzymatic membrane reactors to produce cyclodextrins has been reported starting from different sources including soluble potato and corn starch. A recent work (49) reported also their production using enzyme membrane reactors starting from tapioca starch. The production of oligosaccharides to be used as functional food was also obtained by the immobilization of dextranase on polymeric matrix (50).

The production of substances that preserve the food from contamination or from oxidation is another important field of membrane bioreactor. For example the production of high amount of propionic acid, commonly used as antifungal substance, was carried out by a continuous stirred tank reactor associated with ultrafiltration cell recycle and a nanofiltration membrane (51) or the production of gluconic acid by the use of glucose oxidase in a bioreactor using PES membranes (52). Lactic acid is is widely used as acidulant, flavour additive and preservative in the food, pharmaceutical, leather and textile industries. As an intermediate product in mammalian metabolism, L(+) lactic acid is more important in the food industry than the D(-) isomer. The performance of an improved fermentation system, i.e. a membrane cell-recycle bioreactors MCRB was studied (53)(54), the maximum productivity of 31.5 g/L h was recorded, 10 times greater than the counterpart of the fed-batch fermentation (54).

4.1.2.3. Immobilization of biocatalysts on membranes

The choice of reactor configuration depends on the properties of the reaction system. For example, bioconversions for which homogeneous catalyst distribution is particularly important are optimally performed in a reactor with the biocatalyst compartmentalized by the membrane in the reaction vessel. The membrane is used to retain large components, such as the enzyme and the substrate while allowing small molecules to pass through (e.g the reaction product). For more labile molecules, immobilization may increase the thermal, pH and storage stability of biocatalysts.

Biocatalysts can be entrapped within the membrane, gelified on the membrane surface or bounded to the membrane surface or inner polymeric matrix (55).

The entrapment method of immobilization is based on the localization of an enzyme within a polymer membrane matrix. It is done in such a way as to retain biocatalyst while allowing penetration of substrate.

Asymmetric hollow fibers provide an interesting support for enzyme immobilization, in this case the membrane structure allow the retention of the enzyme into the sponge layer of the fibers by cross-flow filtration. The amount of biocatalyst loaded, its distribution and activity through the support and its lifetime are very important parameters to properly orientate the development of such systems. The specific effect that the support upon the enzyme, however, greatly depend upon both the support and the enzyme involved in the immobilization as well as the method of immobilization used.

The amount of the immobilized biocatalyst is an important parameter and strongly affect the reactor performance, enzymes in fact, are not able to work at high concentration.

The amount of immobilized protein can be determined by the mass balance between initial and final solutions (56). A combined qualitative method merged from the classical *in situ* detection of enzyme activity and western blot analysis can be applied to determine the enzyme spatial distribution through the membrane thickness and along the membrane module and its activity after the immobilization (57)(58)(59).

The gelification of the biocatalyst on the membrane is based on one of the main drawbacks of membrane processes: concentration polarization phenomena. Disadvantages of this systems is the reduction of the catalytic efficiency, due to mass transport limitations and the possibility to preferential pathways in the enzyme gel layer (60).

The bound of the biocatalyst to the membrane can be divided in three principal groups: ionic binding, cross-linking, covalent-linking.

In literature there are various routes to carry enzyme immobilization creating a bound on supports, the principal strategies are based on chemical grafting or molecular recognition on porous supports. The sites involved in this chemistry are generally carboxylic acid, hydroxyls, amino or quaternary ammonium groups, which are created on the surface of porous material by various means like the direct chemical surface treatment or the plasma or UV activation.

The reactive sites thus created allow the attachment of the enzyme by use of coupling reagents such as tosyl chloride, dicyclohexylcarbodiimide, glutaraldehyde etc.

Approaches aiming at creating bio-compatible environments consist in modifying the surface of polymeric membranes by attach of functional groups like sugars, polypeptides and then to adsorb the enzymes.
Another way considered as of bio-mimetic inspiration and which was shown to be efficient for enzyme attachment, it consists in using the very strong and specific interaction of the small protein avidin for the biotin (61)(62). The tetrameric structure of avidin permits itself to interact with four different molecules of biotin at the same time. Various proteins and enzyme could be easily biotinylated, and this mode of enzyme grafting has already been used for electrodes production as well as for membranes made up of conducting fibres.
Although immobilization of enzymes generally enhance their stability, one major disadvantage of random immobilization of enzymes onto polymeric Microfiltration type membrane is that the activity of the immobilized enzymes is often significantly decreased because the active site may be blocked from substrate accessibility, multiple point-binding may occur, or the enzyme may be denaturated (31). Different approaches are developed in order to accommodate site-specific immobilization of enzymes with different structural characteristics, as gene fusion to incorporate a peptidic affinity tag at the N- or C- terminus of the enzyme; post translational modification to incorporate a single biotin moiety on enzymes; and site-directed mutagenesis to introduce unique cysteins to enzymes (63)
The selection of the membrane to be used in enzymatic membrane reactors should take into account the size of the (bio)catalyst, substrates, and products as well as the chemical species of the species in solution and of the membrane itself. An important parameter to be used in this selection is the solute rejection coefficient, which should be zero for the product to facilitate permeation, and should be one for the enzyme to insure a complete retention of the catalyst inside the reaction system. The selectivity is normally associated with a discrimination based on size exclusion, but when a steric exclusion process may be present for molecule with size close to the pore size.

4.1.2. Conclusion

Membrane bioreactors are introduced over 30 years ago, and until now their main industrial application have been for water treatment. In the biotechnology field the development of membrane bioreactors has obtained only partial success. The main technological difficulties in using membrane bioreactors on an industrial level are related with rate-limiting aspects and scale-up difficulties of this technology, together with the life-time of the enzyme, the

availability of pure catalysts at an acceptable cost, the necessity for biocatalysts to operate at low substrate concentration and without microbial contamination.

Many studies are oriented to the investigation of operating conditions and optimization to of the various properties of membrane bioreactors. The efficiency of the overall systems depends on the biochemical (e.g. catalytic activity, reaction kinetics, concentrations, viscosity of substrate, and product immobilization stability), geometric parameters, (membrane configuration, morphology and pore size distribution) and hydrodynamic parameters (such as transmembrane pressure and flow velocity).

4.2. Analysis of patent development in membrane bioreactors from 2004 to 2008

4.2.1. Introduction

The interest towards the development of Membrane Bioreactors (MBR) was analyzed in terms of patent publication from 2004 to 2008 on http://gb.espacenet.com. A list of most recent patents is reported in Appendix 1 (Table 4.2.) and in Table 4.3.

As shown in Fig 4.5. a, there was an increasing development until 2006, followed by an inflexion in recent years.

An accurate analysis of patents published in these four years showed that the main application of Membrane Bioreactors was water treatment, that represent the 77% of the total, while Food and Biotechnology/pharmaceutical fields represent the12%, pollution the 3% and 8% is

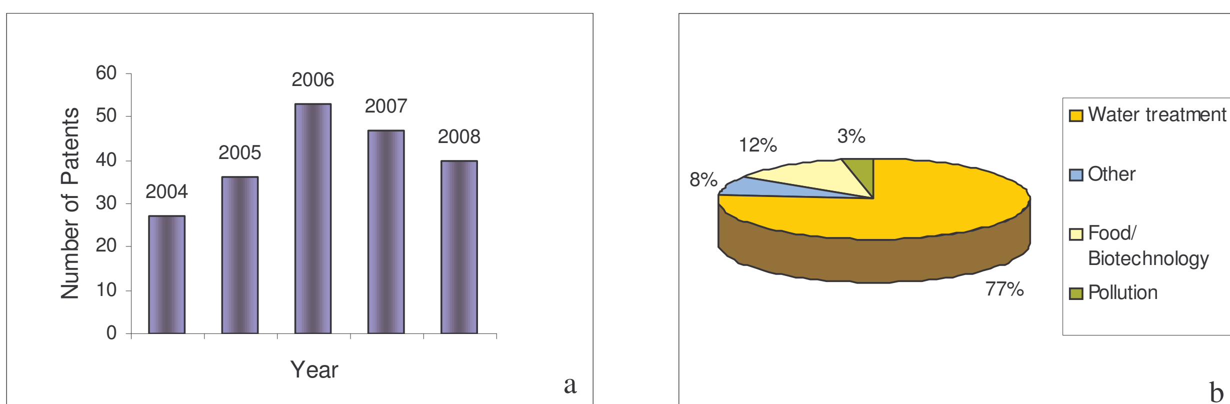


Fig 4.5. a) Number of patent developed from 2004 to 2008 in the field of membrane bioreactors; b) percentage of application of membrane bioreactors in various fields.

represented from other kind of application (Fig 4.5.b). The higher interest in Membrane Bioreactor development for water treatment application was due to the fact that in this moment the need of water is one of the main problem in the world.

In Fig 4.6 the development of Membrane Bioreactors in different countries is reported, while in Fig 4.7 a schematic representation of water in the world is represented in terms of presence/absence of water from the environmental/economical point of view. Comparing the two figures it is possible to see that the development of the Membrane Bioreactors in this field was dictated from the strong demand of water. The countries where the development of MBR is higher are: China followed by United States, where the need of water it due to the shortage of water from the environmental point of view (Fig 4.6).

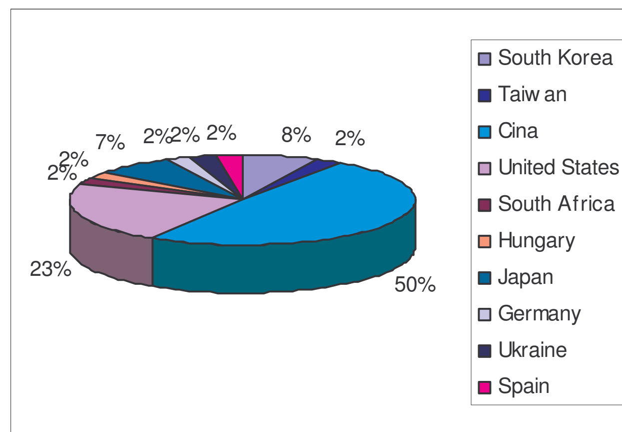


Fig 4.6. Worldwide distribution of patents on membrane bioreactor.

Shortage of water from the environmental point of view
Strong shortage of water from the environmental point of view
Shortage of water from the economical point of view
Low or any shortage of water
Not considered

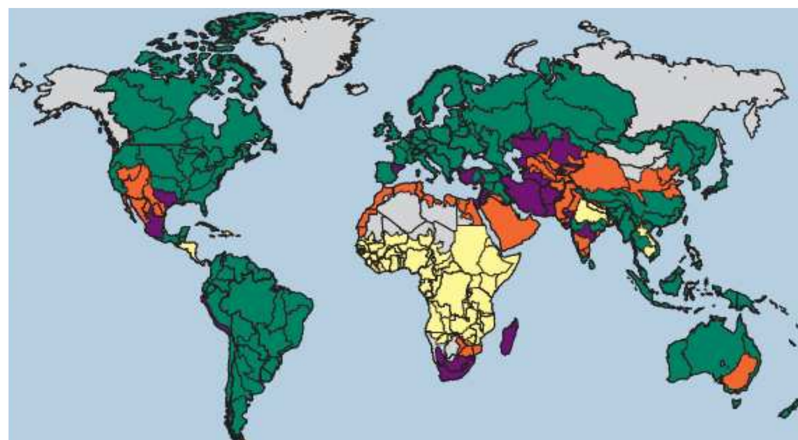


Fig. 4.7. Schematic representation of water content in the world

The research on water treatment refers to: municipal waste water treatment, industrial waste water treatment and drinking water treatment. In Asia and Europe the main water treatment applications using MBR are in the field of municipal waste water, while in North America the main application is industrial waste water. Most of MBR for wastewater treatment are of submerged type.

A wide number of pilot systems using MBR on water treatment are active in different parts of the world, about 15% are collocated in North America, 10% in Europe and 75% in Asia. Zenon Environmental is the main company working in this field in North America, while Kubota, USFilter, Mitsubishi-Rayan have plants worldwide.

4.2.1.2. MBR in Food and Health

Although the application of MBR in food, nutraceutical pharmaceutical and biotechnology is studied since longer time, the patent development has been quite lower compared to water application. A list of recent patents from 2004 to 2008 in Table 4.3 it is reported.

One of the first patent was developed in Japan in 1988 (JP33188386). The aim was the production of a membrane bioreactor for cells growth but adaptable to different kind of systems and for both food and pharmaceutical. Another patent developed in Japan 1991 (JP3039067), was about the production of a yeast product, having good flavour with eliminated odour, taste, peculiar to yeast and suitable as a food material. The yeast culture was carried cut by using a membrane type bioreactor, by culturing yeast fungal cells in a culture medium containing soy sauce and glucose, filtering the resultant culture through a flat membrane and collecting the fungal cells.

A patent developed in Korea in 1997 (KR970003067B) was about the production of Natural antioxidant enzymatic hydrolysate produced by performing hydrolysis in third step enzyme reactor after extracting gelatin using dipping fish-skin in hot water.

A continuous production process of bio-surfactant using enzyme immobilizing technique was developed in Korea in 2001 (KR20010001813). The work is divided in the following steps: (i) manufacturing organic/inorganic mixed membrane where enzyme is immobilized, by sol-gel process; (ii)producing pure malto-oligosaccharide from starch by using a membrane reactor; (iii) immobilizing enzyme physically while agitating enzyme with pre-polymer when manufacturing poly-urethane foam and packing poly-urethane foam at PFR reactor; (iv) supplying malto-oligosaccharide to packed PFR reactor and producing bio-surfactant.

Table 4.3. List of patents on food, nutraceuticals, pharmaceuticals, biotechnology

Title	Inventor	Publication number	Year
1) MEMBRANE ENZYME REACTOR AND METHOD OF PRODUCING BIOPRODUCTS BY USING THE SAME	LEE WEN-CHIEN	TW593676B	2004
2) PROCESS OF CONTINUOUS PRODUCTION OF CASEIN BIOACTIVE PEPTIDE BY ENZYMOLYSIS AND FILTERING MEMBRANE CONCENTRATION	HE ZHIMIN QI	CN1546682	2004
3) ENZYMATICALLY CATALYSED PROCESS	VAN GEMERT ROBERT WILHELM ; CUPERUS FOLKERT PETRUS	US2003068790	2004
4) ULTRAFILTRATION MEMBRANE, DEVICE, BIOARTIFICIAL ORGAN AND METHODS	FISSELL WILLIAM H IV	WO2004024300	2004
5) ENZYMATIC PROCESS FOR OBTAINING ASTAXANTHIN AND PROTEIN FROM FERMENTED SHRIMP RESIDUES	GUERRERO LEGARRETA MARIA ISABE	MXPA02012838	2004
6) DEVICE AND METHOD FOR ENZYMATICALLY PRODUCING BACCATIN III	FRENSE DIETER; LISICKI DOREEN; PFLIEGER CHRISTIAN; LAUCKNER GERALD	WO2005066353	2005
7) COUPLING BIOLOGIC REACTOR	TAO WENYI	CN1560222	2005
8) BIOREACTOR AND METHOD FOR CULTURING CELL USING THE SAME	TSUZUKI HIROHIKO; TODA SATORU	JP2005034069	2005
9) METHOD FOR ENZYMATIC HYDROLYSIS OF FATS/OILS, AND FOR KOMPLEX SEPARATING OF PRODUCTS	BELAFINE DR BAKO KATALIN; NAGY ENDRE DR	HU0401348	2006
10) SIMULTANEOUS PARAMETER EVALUATION DEVICE FOR CELL CULTIVATION PROCESSES	MARX UWE; RIEDEL MARCO; BUSHNAQ-JOSTING HIKMAT	WO2006120202	2006
11) LINEAR AND MEMBRANE-LIKE BIODEVICES AND BIOREACTORS	KONISHI SATOSHI	CA2511457	2006
12) MICROBIOREACTOR FOR CONTINUOUS CELL CULTURE	ZHANG ZHIYU; BOCCAZZI PAOLO ; CHOI HYUN-GOO; JENSEN KLAVS F ; SINSKEY ANTHONY J	US2006199260	2006
13) IMPLANTABLE BIOREACTORS AND USES THEREOF	SHALEV ALON	WO2006080009	2006
14) MICROBIOREACTOR FOR CONTINUOUS CELL CULTURE	ZHANG ZHIYU; BOCCAZZI PAOLO ; CHOI HYUN-GOO ; JENSEN KLAVS F; SINSKEY ANTHONY J	WO2006037022	2006
15) PRODUCTION OF A CONCENTRATED SOLUTION CONSISTING OF BIOLOGICAL SUBSTANCES	FACHHOCHSCHULE GIESSEN FRIEDBE	WO2006005305	2006
16) LINEAR AND MEMBRANE-LIKE BIODEVICES AND BIOREACTORS	KONISHI SATOSHI ; MIWA TETSUYA	CA2511457	2006
17) DRUG AND GENE DELIVERY BY POLYMER NANONOZZLE AND NANOTIP CELL PATCH	LEE L JAMES ; WANG SHENGNIAN ; XIE YUBING ; ZENG CHANGCHUN ; KOH CHEE GUAN ; FEI ZHENGZHENG	WO2007053802	2007
18) MICROBIOREACTOR FOR CONTINUOUS CELL CULTURE	ZHANG ZHIYU ; BOCCAZZI PAOLO; CHOI HYUN-GOO ; JENSEN KLAVS F; SINSKEY ANTHONY J	WO2007038572	2007
19) PROCESS FOR PROMOTING YELLOW WINE FERMENTATION BY TUBULAR ULTRAFILTRATION MEMBRANE REACTOR	JI ZHAOQING	CN1978625	2007
20) CELL EXPANSION APPARATUS WITH PLATE BIOREACTOR	ANTWILER GLEN DELBERT	US2008227190	2008
21) DISPOSABLE TUBING SET FOR USE WITH A CELL EXPANSION APPARATUS	ANTWILER GLEN DELBERT	WO2008109200	2008

AND METHOD FOR STERILE SAMPLING			
22) RETENTION OF COUNTERIONS IN THE SEPARATIVE BIOREACTOR	LIN YUPO JLIN YUPO J	US2008187902	2008
23) IMPLANTABLE BIOREACTORS AND USES THEREOF	SHALEV ALON	US2008112995	2008
24) FILLING BRACKET PERFUSION TYPE BIOREACTOR FOR ARTIFICIAL LIVER	LANJUAN LI	CN101129277	2008
25) MICROCAPSULE SUSPENSION TYPE FLUIDIZED BED TYPE BIOREACTOR FOR ARTIFICIAL LIVER	LANJUAN LI	CN101129276	2008

A patent developed in 2002 in US(US6379922) was about the development of an enzyme membrane reactor designed to enhance the production efficiency of foods, pharmaceutical products, and other bioproducts. The reactor is formed of a cap and a container in which a plurality of the biocatalyst-immobilized sheets are located at an interval. The reactor works in such a way that a liquid reaction mixture is introduced into the container via an inlet of the container, thereby resulting in the synthesis of a bioproduct mixture which is collected via an outlet of the reactor. The immobilized biocatalyst is prepared by entrapping a biocatalyst with a gluten matrix. The immobilized biocatalyst is deposited on supporting meshes contained by a plurality of frames that are inserted in a plurality of pairs of grooves contained by two opposite side walls of the container. Deactivated biocatalyst-immobilized sheets are replaceable and recyclable.

The work in the patent (2) relates to a process of continuous production of casein bioactive peptide by enzymolysis and filtering membrane concentration which comprises, using caseinum as raw material and at least one prolease action, obtaining biologically active polypeptides having multiple functions in multi-stage enzyme membrane reactor combined from enzymolysis tank and hyperfiltration, nano filter membrane of dissimilar entrapment molecular weight.

An enzymatic membrane reactor with lipase (as preferred enzyme) immobilized on membrane was developed in patent (3) to remove water produced during reaction.

The invention (6) relates to a device for enzymatically producing baccatin III in an enzyme reactor. The enzyme reactor is formed by a hollow fiber ultrafiltration membrane, and an acetyl transferase from Taxus spec. immobilized inside the reactor. The invention also relates to a method for enzymatically producing baccatin III from 10-deacetylbaccatin III (10-DAB). The method enables the production of baccatin III without requiring complicated purification steps of the acetyl transferase. The purification of the baccatin III is also simplified.

A method of producing fatty acids by enzymatic hydrolysis of vegetable or animal oils or fats in a membrane biological reactor of flat x-plate system was developed in patent (9). The system provides flowing conditions near to turbulent in the biological reactor by canals and

deflectors formed in the internal surface and removing by dialysis the glycerin which is produced continuously during the reaction.

Patent (17) reports about delivery of drugs or genes to individual cells achieved on a nanoscale using electroporation techniques. In one method, a flow-through bioreactor having an inlet and an outlet connected by a flow chamber and a nanoporous membrane positioned in the flow chamber is used. Cells to be electroporated are flowed from the inlet to the outlet, a quantum of molecules of the at least one drug or gene in a fluid medium in the flow chamber.

In Table 4.4 are summarized some of the most recent applications of enzyme in food that could be used to implement new enzyme membrane reactor development.

Table 4.4 Enzymes used in food

Enzyme	Application	Patent number	Year
CELLULOLYTIC ENZYME	BREAD AND BAKERY PRODUCTS HAVING ANTIMICROBIAL PROPERTIES	RU2316215	2008
PECTINASE AND CELLULASE	PRODUCING SOFT VEGETABLE MATERIAL	WO2008029783	2008
AMYLASE	CAN REMOVE TRYPSIN INHIBITORS KNOWN AS ANTI-NUTRITIONAL FACTORS FROM FERMENTED SOYBEAN PRODUCTS	US2008044501	2008
ANGIOTENSIN CONVENING ENZYME INHIBITOR (ACEI)	PRODUCTION OF KIND OF FOOD ADDITIVE	US2008044503	2008
AMYLASE, LIPASE AND ASCORBIC ACID OXIDASE; AND HEMICELLULASE	USED AS ACTIVE INGREDIENTS TO OBTAIN A SAFE QUALITY IMPROVER FOR BREAD ON FOOD SANITATION	JP2007325515	2008

Appendix 1:

Table 4.2: List of patent published on MBR from 2006 to 2008 (listed in chronological order)

Title	Inventor	Publication number	Year
COMPOSITE MEMBRANE BIOREACTOR	YANG JIANZHOU ZHANGFAN	CN1785829	2006
INTEGRATED TUBULAR DYNAMIC MEMBRANE BIOREACTOR	FAN YAOBO WU	CN1789167	2006
TUBULAR DYNAMIC MEMBRANE REACTOR FOR BIOLOGICAL CATALYTIC OXIDATION	FAN YAOBO DONG	CN1789166	2006
TREATMENT OF BIOLOGICAL REACTOR FOR COKING SEWAGE MEMBRANE	YIN JUNXIAN ZHANG	CN1778726	2006
A METHOD FOR IMPROVING FLUX IN A MEMBRANE BIOREACTOR	YOON SEONG-HOON	ZA200506536	2006
MEMBRANE BIOREACTOR	KUO JOSEPH	US2006049092	2006
COMPOSITE AERATION TYPE MEMBRANE BIOREACTOR	LIAO ZHIMIN LI	CN1884131	2006
MEMBRANE BIOREACTOR	GUO XINGZHONG	CN1727289	2006
WASTE GAS TREATMENT EQUIPMENT EMPLOYING SCRUBBER AND MEMBRANE BIOREACTOR AND METHOD THEREOF	XU SHUGANG HE	CN1864810	2006
SYSTEM FOR TREATING WASTEWATER CONTAINING ORGANIC COMPOUNDS	YOU HUEY-SONG	US2006243661	2006
LOW POWER MEMBRANE BIOREACTOR	SUN BAOSHENG ZHANG	CN1884130	2006
CONTEMINATED INFLOW TREATMENT WITH MEMBRANE DISTILLATION BIOREACTOR	FANE ANTHONY	WO2006137808	2006
MEMBRANE BIOREACTOR PROCESS AND AERATOR	LIU MINGGANG	US2006094748	2006
MEMBRANE TYPE BIOREACTOR AND METHOD FOR TREATING LIQUID BY USING THE SAME	EGUCHI MASAHIRO	JP2006094748	2006
EXTERNAL-SUBMERSED MEMBRANE BIOREACTOR WITH MINIMIZED AIR SCRUBBING OF MEMBRANE MODULE	CHAE KYU JUNG	KR100649261B	2006
LOW-POWER MEMBRANE BIOREACTOR	SUN BAOSHENG ZHANG	CN1884130	2006
TWO SECTION TYPE DYNAMIC MEMBRANE BIOREACTOR	ZHANG JIAN QIU	CN2848840Y	2006
INTERNAL CIRCULATION DYNAMIC MEMBRANE BIOREACTOR	ZHANG JIAN QIU	CN1872733	2006
WASTE GAS TREATMENT EQUIPMENT EMPLOYING SCRUBBER AND MEMBRANE BIOREACTOR AND METHOD THEREOF	XU SHUGANG HE	CN1864810	2006
THE APPARATUS AND THE METHOD FOR THE VOC AND ODOR REMOVAL USING A HOLLOW FIBER MEMBRANE BIOREACTOR	SONG JI HYEON	KR100558628B	2006
BACK-FLUSHING OPERATION METHOD OF DYNAMIC MEMBRANE BIOREACTOR	ZHANG JIAN QIU	CN1843971	2006
SYSTEM FOR DETECTING MEMBRANE MODULE INTEGRITY OF MEMBRANE BIOREACTOR	ZHU GAOXIONG DING	CN1844889	2006
METHOD FOR TREATING WASTEWATER IN A MEMBRANE BIOREACTOR TO PRODUCE A LOW PHOSPHORUS EFFLUENT	DAIGGER GLEN T FLEISCHER EDWIN	CN1845879	2006
ENERGY-SAVING INTEGRATED TYPE MEMBRANE BIOREACTOR	ZHANG HAIFENG QI	CN1837086	2006
METHOD FOR CLEANING A SEPARATION MEMBRANE IN A MEMBRANE BIOREACTOR SYSTEM	KANDO KOICHIRO	EP1704911	2006
SLUDGE OUTER CIRCULATION TYPE SEWAGE TREATMENT METHOD OF DENITRIFYING AND RECOVERING PHOSPHOR USING COMPOSITE MEMBRANE BIOREACTOR	ZHANG LIQIU YUAN	CN1807276	2006
COMPOSITE MEMBRANE BIOREACTOR	YANG JIANZHOU ZHANGFAN	CN1785829	2006
SPLIT-TYPE TUBE TYPE DYNAMIC MEMBRANE BIOREACTOR	FAN YAOBO LI	CN1785841	2006
ON-LINE CHEMICAL CLEANING METHOD FOR MEMBRANE BIOREACTOR	HUANG XIA WEI	CN1772355	2006
COMBINED MEMBRANE BIOREACTOR-REVERSE OSMOSIS UNIT FOR TREATING NON-DEGRADABLE INDUSTRIAL EFFLUENT	DING HUANRU WANG	CN1772649	2006
BIOREACTOR PROCESSING METHOD WITHIN A TANK INTERNALLY CHAMBERED TO SEQUENTIALLY PERFORM BIOLOGICAL TREATMENT AND MEMBRANE FILTRATION	KULICK III FRANK M	US7052607	2006
HOLLOW FIBER MEMBRANE TYPE BIOREACTOR AND LIQUID TREATMENT METHOD USING THE SAME	ERA AKIRA; EGUCHI MASAHIRO	JP2006101805	2006
MEMBRANE TYPE BIOREACTOR AND METHOD FOR TREATING LIQUID BY USING THE SAME	EGUCHI MASAHIRO; ERA AKIRA	JP2006094748	2006
MEMBRANE-TYPE BIOREACTOR AND LIQUID TREATMENT METHOD USING THE SAME	HATANAKA CHIAKI; EGUCHI MASAHIRO; ERA AKIRA	JP2006087310	2006
MEMBRANE BIOREACTOR, PROCESS AND AERATOR	LIU MINGGANG	US2006054552	2006
MEMBRANE BIOREACTOR WASTE WATER TREATMENT METHOD	LANGLAIS CHRYSTELLE	PL376562	2006
MARINE VESSEL ONBOARD WASTEWATER TREATMENT SYSTEM	HIGGINS JAMES E	US7108782	2006
SOLAR ENERGY MEMBRANE BIOREACTOR	LU JINXI LU	CN1765766	2006
INTEGRATED TUBULAR DYNAMIC MEMBRANE BIOREACTOR	FAN YAOBO WU	CN1789167	2006
THROTTLE VALVE SEPARATED TYPE MEMBRANE BIOLOGIC REACTOR	FAN YAOBO XU	CN1861530	2006
FILTRATION APPARATUS COMPRISING A MEMBRANE BIOREACTOR AND A TREATMENT VESSEL FOR DIGESTING ORGANIC MATERIALS	JORDAN EDWARD J	US2006201876	2006
PROCESS FOR REDUCING FILM POLLUTION OF PLATE FILM-FILM BIOLOGICAL REACTOR	WU ZHICHAO YANG	CN1724410	2006
ROTARY DISK TYPE MEMBRANE BIOREACTOR AND ITS WATER TREATMENT METHOD	XU YOUYI ZUO	CN1769195	2006
METHOD AND APPARATUS FOR CLEANING EFFLUENT	DOBIE KEITH	US6998048	2006
WASTEWATER TREATMENT SYSTEM AND METHOD OF TREATING WASTEWATER	JOHNSON BRUCE R	US2006113244	2006

METHOD AND DEVICE FOR TREATING MICROORGANISM-CONTAINING EFFLUENTS	LANGLAIS CHRYSTELLE	WO2007063198	2007
OPTICAL BIOREACTOR FOR SPACING REGENERATIVE OXYGEN	LIU HONG YANG (CN)	CN101033449	2007
BIOLOGICAL WASTE WATER TREATMENT REACTOR HAS CROSS-FLOW BIO-MEMBRANE FILTER LOCATED IMMEDIATELY BEFORE REACTOR OUTLET	AQUADETOX INTERNAT	DE102006001603	2007
METHOD FOR WASTEWATER TREATMENT BY MEANS OF BIOREACTOR OF MEMBRANE TYPE	XU YOUYI SHEN	UA80724	2007
COMB-LIKE MEMBRANE-BIOREACTOR HAVING FREE-END	XU YOUYI SHEN	CN101062809	2007
SEPARATOR AND IMMERSION MEMBRANE BIOREACTOR COMBINED WATER PURIFYING DEVICE AND SYSTEM	ZHANG MINLIANG	CN101033107	2007
INTENSIFIED ANTI-NITRATED PHOSPHOROUS-REMOVAL SEQUENCING BATCH MEMBRANE BIOREACTOR TECHNIQUE	ZHANG HANMIN YANG	CN101062807	2007
APPARATUS AND METHOD FOR TREATING SEWAGE BY AIR-LIFTING INTERNAL CIRCULATING MEMBRANE BIOREACTOR	YE YAPING ZHANG	CN1935689	2007
AREATED ANOXIC MEMBRANE BIOREACTOR	BARNES DENNIS J	US2007235385	2007
MEMBRANE BIOREACTOR AND METHOD FOR THE BIOLOGICAL TREATMENT OF OILY WASTEWATER	SCHAIBLE JUERGEN	EP1803688	2007
METHOD OF INHIBITING MEMBRANE CONTAMINATION OF MEMBRANE BIOREACTOR	CHEN JIAAN LI	CN1974439	2007
MEMBRANE BIOREACTOR	LI LIANKUN	CN1944282	2007
WASTEWATER TREATING APPARATUS WITH CIRCULATING MEMBRANE BIOREACTOR	LIU JUNXIN LI	CN1974437	2007
GARBAGE LEACHATE PROCESSING PROCESS AND SYSTEM BASED ON MEMBRANE BIOREACTOR-NANO FILTERING MEMBRANE TECHNOLOGY	LIAO ZHIMIN GUO	CN1970474	2007
BIOREACTOR OF DYNAMIC MEMBRANE OF 3D FILTER CLOTH	ZHANG JIAN QIU	CN1915857	2007
ABSORPTION REGENERATION DETACHABLE MEMBRANE BIOREACTOR FOR TREATING WATER	FANGZHEN REN; JIAOSHI WU; GUOXUN XU	CN101190807	2007
MEMBRANE BIOREACTOR USING NON-WOVEN FABRIC FILTRATION	CHANG WANG-KUAN; CHANG MIN-CHAO; HORNG REN-YANG; SHAO HSIN	TW284120B	2007
APPARATUS AND PROCESS FOR TREATING WASTE GAS BY USING A SCRUBBER AND MEMBRANE BIOREACTOR	HSU SHU-KANG (TW); HO TSUNG-JEN (TW); LIU WEN-TZONG (TW); CHANG REY-YUE (TW)	TW283189B	2007
DYNAMIC CONTROL OF MEMBRANE BIOREACTOR SYSTEM	ZHA FUFANG; LIU WENJUN	AU2006299746	2007
MEMBRANE CLEANING AND DISINFECTION METHOD FOR MEMBRANE BIOREACTOR USED FOR E.G. WATER PURIFICATION, COMPRISES FORMING CLEANING AGENT BY ELECTROLYSIS AND RINSING MEMBRANE VIA BACK WASH TECHNIQUE	NIJMAN NIELS	NL1031936C	2007
EXTERNAL MEMBRANE BIOREACTOR SYSTEM FOR SEWAGE TREATMENT AND METHOD THEREOF	XIE MENG	CN101041510	2007
APPARATUS HAVING A BIOREACTOR AND MEMBRANE FILTRATION MODULE FOR TREATMENT OF AN INCOMING FLUID	FUTSELAAR HARRY, BORGERINK ROB	WO2007139374	2007
INTEGRAL MULTIFUNCTIONAL MEMBRANE BIOREACTOR	ZHANG CHUANYI YUAN	CN200946120Y	2007
METHOD FOR REDUCING MEMBRANE FOULING IN THE MEMBRANE BIOREACTOR BY USING BIVALENT CATION, AND MEMBRANE BIOREACTOR USING THE METHOD	KIM IN SU, JANG NAM JUNG, YIM SEONG KEUN	KR100714366B	2007
COLD ROLLING OIL-CONTAINING WASTE WATER MEMBRANE BIOREACTOR AND ITS PROCESSING METHOD	XIAO BINGYAN CAO	CN1970469	2007
GREASE AND SCUM REMOVAL IN A FILTRATION APPARATUS COMPRISING A MEMBRANE BIOREACTOR AND A TREATMENT VESSEL FOR DIGESTING ORGANIC MATERIALS	JORDAN EDWARD J ELEFRITZ ROBERT	US2007084791	2007
DEVICE OF MEMBRANE BIOREACTOR FOR TREATING GARBAGE PERCOLATION LIQUID	HAN DEMIN WANG	CN2878369Y	2007
FILTRATION APPARATUS COMPRISING A MEMBRANE BIOREACTOR AND A TREATMENT VESSEL FOR DIGESTING ORGANIC MATERIALS	JORDAN ED	EP1747058	2007
BIOREACTOR AND METHOD FOR THE BIOLOGICAL PURIFICATION OF WATER	ZAITSEV GENNADI	CA2624596	2007
APPARATUS AND METHOD FOR TREATING FGD BLOWDOWN OR SIMILAR LIQUIDS	PEETERS JEFFREY GERARD	CA2615945	2007
BIOLOGICAL REACTOR OF POLYGONAL LINE FLOWING FILM	YABIN JIN	CN101092270	2007
TECHNOLOGICAL PROCESS OF TREATING PAPERMAKING EFFLUENT FOR REUSE BASED ON MEMBRANE INTEGRATING TECHNIQUE	YUHAI CHEN	CN101088941	2007
INTEGRAL ANAEROBIC FILM-BIOLOGIC REACTOR-NATURAL VENTILATING BIOLOGIC FILTER POOL SEWAGE TREATING TECHNIQUE	WU ZHICHAO ZHOU	CN101037270	2007
EQUIPMENT AND METHOD FOR TREATING WASTEWATER DIFFICULT TO BE DEGRADED BY BIOLOGY METHOD OF HYDROLYSIS COMPOSITE MEMBRANE	WANG SHIHE WU	CN101050042	2007
METHOD FOR PRE-TREATING SEAWATER BY MEMBRANE BIOLOGICAL REACTOR	CHEN LI XU	CN101028961	2007
CAST-SAND MEMBRANE BIOLOGICAL REACTOR	FAN YAOBO XU	CN101028949	2007
LIGHT BIOREACTOR	WANG XUEQING HAN	CN101003781	2007
A METHOD FOR IMPROVING FLUX IN A MEMBRANE BIOREACTOR	YOON SEONG-HOON	SG128537	2007
METHOD OF CLEANING MEMBRANE IN MEMBRANE SEPARATION ACTIVATED-SLUDGE PROCESS	NOGUCHI MOTOHARU	EP1852175	2007
WASTEWATER TREATMENT SYSTEM AND METHOD	SMITH DUANE R	US2007209999	2007
WASTEWATER TREATMENT SYSTEM AND METHOD	SMITH DUANE R	WO2007103409	2007
MEMBRANE BIOREACTOR	TAYLOR ANTHONY PATRICK ANDREW	KR20070053662	2007
BIOREACTOR-DIRECT MICROBE FUEL CELL AND USE THEREOF	LIU ZHIDAN LI	CN1949577	2007
CONTACT OXIDIZING H CIRCULATION PIPE FILM BIOLOGICAL REACTOR	FAN YAOBO DONG	CN1899979	2007

MEMBRANE BIOREACTOR HAVING SINGLE HEADER MEMBRANE MODULE	LIU MINGGANG	US2008000832	2008
HYBRID AERATION MEMBRANE BIOREACTOR	LIAO ZHIMING	US2008003669	2008
MEMBRANE BIOREACTOR	TAYLOR ANTHONY P A	US2008044850	2008
PROCESS FOR THE PRODUCTION OF BIOMASS FEED FOR BACTERIA AND USE THEREOF IN A BIOGAS PLANT	BUERGER ADAM (DE)	EP1925661	2008
INTEGRATED WATER PURIFICATION PROCESSING METHOD AND DEVICE OF ULTRAFILTRATION MEMBRANE COAGULATION/BIOREACTOR	GUIBAI LI ; JIAYU TIAN ; YANLING YANG ; HENG LIANG ; XING LI ; JIE CHEN	CN101219847	2008
ULTRAFILTRATION MEMBRANE COAGULATION /ADSORPTION/BIOREACTOR INTEGRATING ADVANCED WATER TREATMENT METHOD AND DEVICE	GUIBAI LI (CN); JIAYU TIAN (CN); HENG LIANG (CN); XING LI (CN); JIE CHEN (CN)	CN101219846	2008
HIGH ROAD SERVICE AREA SEWAGE WATER TREATING PROCESS AND DYNAMIC MEMBRANE BIOREACTOR	DAFANG FU; RONG NIE ; QIANG MA ; JIE LI ; XIN HUI ; QING ZHOU	CN101200341	2008
METHOD FOR PRODUCING RECLAIMED WATER BY MEMBRANE BIOREACTOR-OZONE COMBINATION TECHNIQUE	JIANPING WEN ; HUI LIANG ; JIANZHOU YANG; YILI CHEN	CN101200331	2008
DYNAMIC CONTROL OF MEMBRANE BIOREACTOR SYSTEM	LIU WENJUN; ZHA FUFANG ; KUZMA MATTHEW ; JORDAN EDWARD JOHN	CA2622930	2008
METHOD FOR PRODUCING BIFIDOBACTRIUM SP. STRAINS IN A HIGH YIELD USING SUBMERGED MEMBRANE BIOREACTOR	OH DEOK KUN; KWON SOUN GYU; SON JU WAN	KR20080026387	2008
MEMBRANE-CLEANING METHOD FOR MEMBRANE BIOREACTOR PROCESS	Not specified	US2008105615	2008
INFILTRATION/INFLOW CONTROL FOR MEMBRANE BIOREACTOR	SMITH GEORGE W; BARNES DENNIS J	WO2008051546	2008
MEMBRANE BIOREACTOR FOR PHOSPHORUS REMOVAL	DALE DARREN ; GALLAGHER PAUL M	WO2008048594	2008
CONTAMINATED INFLOW TREATMENT WITH MEMBRANE DISTILLATION BIOREACTOR	FANE ANTHONY G ; PHATTARANAWIK JIRACHOTE ; WONG FOOK-SIN	EP1909948	2008
METHOD OF IMPROVING MEMBRANE BIOREACTOR PERFORMANCE	YOON SEONG-HOON,COLLINS JOHN H	US2008060996	2008
MEMBRANE BIOREACTOR	TAYLOR ANTHONY P A ; FINNIE KIM S ; BARTLETT JOHN ; HOLDEN P J	US2008044850	2008
ANAEROBIC COMPOSITE TYPE MEMBRANE BIOREACTOR COMBINED SYSTEM AND METHOD FOR TREATING MIXED WASTE WATER	DAN HAN ; FEIYU HONG ; PIN GAO; XIAOJING ZHAN; YONGYUN XIA	CN101121566	2008
INTEGRATIVE AEROBIC PARTICLE SLUDGE MEMBRANE BIOREACTOR SEWAGE TREATMENT METHOD	JINGFENG WANG ; JUNWEN LI ; XUAN WANG ; ZHAOLI CHEN ; MIN JIN ; ZHIQIANG CHEN ; ZHIGANG QIU ; XINWEI WANG ; JING YIN	CN101100334	2008
BATCH TYPE AEROBIC PARTICLE SLUDGE MEMBRANE BIOREACTOR SEWAGE TREATMENT TECHNIQUE	SHUGUANG WANG ; WENXIN GONG; XUEFEI SUN ; XIANWEI LIU ; CHENGLU ZHANG	CN101100333	2008
METHOD FOR WASTEWATER TREATMENT BY MEANS OF BIOREACTOR OF MEMBRANE TYPE	Not specified	UA80724	2008
COMPOSITE MEMBRANE BIOREACTOR	BAOZHEN WANG ; LIN WANG ; SHUO LIU; SHUMEI WANG ; JIN WANG ; XIANGDONG CAO ; DAI WANG ; LI WANG	CN200999217Y	2008
MEMBRANE BIOREACTOR HAVING SINGLE HEADER MEMBRANE MODULE	LIU MINGGANG ; HUSAIN HIDAYAT	US2008000832	2008
HYBRID AERATION MEMBRANE BIOREACTOR	LIAO ZHIMING ; LI RONG; JU DEJIN); WU JIJUN ; GUO JINGKUI; HUANG YUHE ; CAI DONGSHENG ; WAN AIGUO ; YU KUN ; LIU ZHIZHONG	US2008003669	2008
HIGH PERFORMANCE, ENERGY EFFICIENT SYSTEM AND METHOD FOR WASTEWATER TREATMENT WITH RESOURCE RECOVERY AND REDUCED RESIDUAL SOLIDS GENERATION	SUTTON PAUL M	WO2008115444	2008
APPARATUS AND METHOD FOR THE TREATMENT OF WASTE	AMES ROY VICTOR; ETHERINGTON MATTHEW JOHN; CZEPERKO NICHOLAS MICHAEL; UGARTE IAN JOSEPH	NZ565549	2008
DRAIN-FLUSH SEQUENCE AND SYSTEM FOR FILTER MODULE	MORGAN CHARLES R, LEBRUN LOUIS E, BALL ANDREW	WO2008094456	2008
TREATMENT OF WASTEWATERS USING DUAL-STAGE MEMBRANE BIOREACTORS	EDWARDS WADE	WO2008093287	2008
SYSTEM AND METHOD FOR TREATING WASTEWATER	FABIYI MALCOLM ; NOVAK RICHARD	WO2008042761	2008
TREATING METHOD OF ENVIRONMENTAL POLLUTANT AND BIOREACTOR	UEMOTO HIROAKI	JP2008049334	2008
METHOD FOR TECHNOLOGICAL WATER TREATMENT	PAPP ISTVAN	HU0500225	2008
METHOD FOR SUPPLYING ELECTRON DONOR TO MICROORGANISM AND ITS DEVICE, AND BIOREACTOR USING IT	UEMOTO HIROAKI; MIMURA HAJIME; YOSHIDA TATSUNORI	JP2008023488	2008

WASTEWATER TREATMENT SYSTEM AND METHOD OF TREATING WASTEWATER	JOHNSON BRUCE R	US2008047896	2008
A METHOD FOR IMPROVING FLUX IN A MEMBRANE BIOREACTOR	YOON SEONG-HOON	EP1885656	2008
METHOD AND APPARATUS FOR TREATMENT OF WASTEWATER EMPLOYING MEMBRANE BIOREACTORS	ARNAUD JOHNNY	US2008006571	2008

Source: Esp@senet, period: from 2004 to August

References

(1) R. A. Sheldon, J. Chem. Tech. Biotechnol., 1997, 68, 381.

(2) M. Pastore, F. Morisi, Methods Enzymol. 44, 1976, 822-830.

(3) W. Carasik, J. O. Carrol Food Technol. 37, 1983, 85-91.

(4) J. Bryjak, M. Bryjak, A. Noworyta, Enzyme and Microbial Technology 19, 1996, 196-201.

(5) T. Sato, T. Tosa, In Industrial Application of Immobilized Biocatalyst, Marcel Dekker, 1993, 3-14.

(6) I. Chibata et al., Appli Miocrobiol., 27,1974, 878-885.

(7) S. Takamatsu, Eur. J.Appl. Microbiol. Biotechnol. , 15, 1982, 147-149.

(8) C. Wandrey, E. Flaschel, Advances in Biochemical Engineering, Springer Verlag, Berlin, 12 (1979) 147.

(9) C. Wandrey, R. Wichmann, W. Leuchtenberger, M.R. Kula, US Patent No. 4,304,858 (1981), Degussa AG/GBF.

(10) C. Wandrey, Biochemical reaction engineering for redox reactions, The Chemical Record, 4 2004, 254-265.

(11) I. Takata, T. Tosa, In Industrial Application of Immobilized Biocatalysts, Marcel Dekker, 1993, 53-55.

(12) I. Alkorta, C. Garbisu, M. J. Llama, J. L.Serra, Process Biochemistry 33, 1998, 21-28.

(13) L. Giorno et al. Fruit Processing Sep. Sci. Technol. 33, 1998, 739-756.

(14) K. Oyama, Journal of Chemical Society, 11, 1974, 356-360.

(15) J. L. Lopez, S. Matson, Journal of Membrane Science, 1997, 125 189-211.

(16) S. L. Matson, Patent No. US4800162, 1989.

(17) H. Z. Qi, Patent No CN1546682, 2004.

(18) A. Trusek-Holownia, Biochemical Engineering Journal 39, 2008, 221–229.

(19) L. M. I . Guerrero, patent No MXPA02012838, 2004.

(20) D. Frense, patent NoWO2005066353, 2005.

(21) K. Belafine Bako, patent No HU0401348, 2006.

(22) K. Satoshi M.Tetsuya, patent No CA2511457, 2006.

(23) A. Shalev, patent No WO2006080009, 2006.

(24) A. Shalev, patent No US2008112995, 2008.

(25) Z. Zhang, P. Boccazzi, H. Choi, K. F Jensen. A. Sinskey, patent No.US2006199260, 2006.

(26) D. Antwiler Glen, WO2008109200, 2008.

(27) P. Czermak ; D. Nehring patent No WO2006005305, 2006.

(28) L. J. Lee, S. Wang, Y. Xie, Zeng C., C.G. Koh, Z. Fei, Patent No WO2007053802, 2007.

(29) L. Giorno, E. Drioli, 18 (2000) 339-348.

(30) L. Giorno, N. Li, E. Drioli, Biotech. & Bioeng., 84, 2003, 677.

(31) C. Charcosset, Biotechnology Advances, 24, 2006, 482-492,

(32) C. H. Nam S. Furusaki In: Membrane bioreactors: Present and prospects, Advances in Biochemical Engineering/Biotechnology 44 1991, 27-64

(33) L. Giorno, E. D'Amore, R. Mazzei, E. Piacentini, J. Zhang, E. Drioli, R. Cassano, N. Picci, J. Membr. Sci. 295, 2007, 95-101.

(34) W. Yang, N. Cicek, J. Ilg, J. Membr. Sci, 270, 1997 201-211.

(35) A. K. Goulas, J. M. Cooper, A. S. Grandison, R. A. Rastall, Biotechol. Bioeng. 88, 2004, 778-787.

(36) M. P. Belleville, P. Lonzano, J. L. Iborra, G. M. Rios, Sep Pur. Technol. 25,2001, 229-233.

(37) T. P. Chung., P. C. Wu, R. S. Juang, 258, 2005, 55-63.

(38) L. Giorno, E. Piacentini, R. Mazzei, E. Drioli, J. Membr. Sci, 317, 2008,19-25.

(39) N. Li, K. Sakaki J. Membr. Sci. 314, 2008, 183-192.

(40) Z. Wang , Z. Wu , S. Maib, C. Yang, X. Wang a, Y. Ana, Z. Zhoua, Separation and Purification Technology 62, 2008, 249–263.

(41) T. Gumı, J. Fernandez-Delgado Albacete, D. Paolucci-Jeanjean , M. Belleville, G. M. Rios J Membr. Sci. 311, 2008, 147–152.

(42) E. Pomier, N. Delebecque, D. Paolucci-Jeanjean M. Pina S. Sarrade, G. M. Rios, J. of Supercritical Fluids, 41, 2007 380–385.

(43) A. Boontawan, D. C. Stuckey, Appl Microbiol Biotechnol, 69, 2006, 643–649.

(44) A. Trusek-Holownia, A. Noworyta , Journal of Biotechnology, 130, 2007, 47–56.

(45) S. Novalin, W. Neuhaus , K. Kulbe, Journal of Biotechology 119, 2005, 212-218 .

(46) A. Krastanov, D. Blazheva , V. Stanchev, Process Biochemistry 42, 2007, 1655–1659.

(47) S. Kwona, S. Park, D. Oh, J. of Biosci. and Bioeng., 101, 2006, 13-18.

(48) K. Prochaskaa,, P. Kedzioraa, J. Le Thanhb, G. Lewandowicz, Food Hydrocolloids, 21, 2007, 654–659.

(49) A. M. M. Sakinaha, A. F. Ismaila, R. Md Illiasa, O. Hassand, Desalination 207, 2007, 227-242.

(50) C. Torras, D. Nabarlatz, G. Vallot, D. Montané, R. Garcia-Valls, Chemical Engineering Journal, 144, 2008, 259–266.
(51) P. Boyaval, C. Corre, Lait, 75, 1995, 453-461.
(52) J. Liu, Z. Cui , Journal of Membrane Science, 302, 2007, 180–187.
(53) L. Giorno, K. Chojnacka, L. Donato, E. Drioli, Ind. Eng. Chem. Res., 41, 2002, 433-440.L.
(54) G. Xu, J. Chu, Y. Wang, Y. Zhuang, S. Zhang , H. Peng, Process Biochemistry, 41, 2006, 2458–2463
(55) H. Strathmann, L. Giorno, Drioli E., CNR, Rome, 2006.
(56) L. Giorno et al. J.Chem. Technol Biotechnol,. 64, 1995, 345-352.
(57) S. Mazzuca, L. Giorno, A. Spadafora, R. Mazzei, E. Drioli, J. Membr. Sci. 285 (2006) 152–158.
(58) J. P. S. G. Crespo, M. Trotin, D. Hough, J. A. Howell, J. Membr. Sci. 155 (1999) 209.
(59) Z. M. Liu, J. Dubremez, V. Richard, Q. Yang, Z.K. Xu, P. Seta, J. Membr. Sci. 267 (2005) 2.
(60) E. Drioli, G. Iorio, Edt. Mark C.P. Noyes Publications Park Ridge, New Jersey, USA , 401-481 1989
(61) M. Amounas, C. Innocent, S. Cosnier, P. Seta, J. Memb. Sci. 176, 2000, 169-176.
(62)M. Amounas, V. Magne, C. Innocent, E. Dejean, P. Seta. Enzyme and Microbial Technology 31, 2002, 171-178.
(63) D.A. Butterfield, D. Bhattacharyya, S. Daunert., L. Bachas, J. Membr. Sci., 181, 2001, 29-37.

Chapter 5

Improvement of β-glucosidase activity of *Olea europaea* fruit extracts processed by membrane technology

Abstract

The β-glucosidase from olive fruit is of particular interest compared to the ones from other sources because it has shown to have high specifity to convert the oleuropein to into dialdehydes, which have antibacterial activity and are of high interest for their application in the food and pharmaceutical fields. The enzyme is not yet commercially available and advanced clean and safe technologies for its purification able to maintain the functional stability are foreseen. The purification of this protein from fruit extracts has been already tempted by electrophoresis but either enzyme deactivation or high background with unclear profiles occurred. In this work, fruit extracts obtained from the ripening stage that showed the highest enzyme activity have been processed by diafiltration and ultrafiltration. Asymmetric membranes made of polyamide or polysulphone having 50 and 30 kDa molecular weight cut-off, respectively, were tested for the diafiltration process. 4 kDa polyethersulfone ultrafiltration membranes were used to concentrate the diafiltered permeate solutions. The efficiency of the separation processes was evaluated by enzyme activity tests using the hydrolysis of *p*-D-nitrophenyl-β-D-glucopyranoside (pNPGlc) as reaction model. Qualitative electrophoresis were applied to analyze the composition of protein solution before and after the membrane separation; in addition dot blot and western blot analyses were applied to verify the presence of β-glucosidase in the processed fractions.

The overall results showed that the β-glucosidase functional stability was preserved during the membrane operations and the removal of 20 kDa proteins allowed to increase the specific activity of the enzyme of about 52% compared to the one present in the initial fruit extract.

5.1 Introduction

In the *Oleaceae* family a β-D-glucoside glucohydrolase EC 3.2.1.21 referred to as β-glucosidase, is a key enzyme in many processes such as the defense against pathogens [1], the physiological role during fruit ripening [2, 3] and, restricted to olive tree, the influence in the foodstuff quality resulting from industrial processing of fruits [4, 5]. In addition, the products of enzymatic hydrolysis of natural substrate oleuropein are well known as a pharmacologically active molecule [6]. As a general rule, this enzyme family catalyzes the hydrolysis of glycosidic linkages in aryl and alkyl β-glucosides and cellobiose as natural substrates [7]. However, the assay of activity of β-glucosidase toward the synthetic substrate *p*-D-nitrophenyl-β-D-glucopyranoside (*p*NPGlc) was widely employed in higher plants [8, 9, 10]. In the olive tree the *p*NPGlc substrate was employed to identify β-glucosidase behavior during fruit ripening [11] and in response to the injury [12]. Drupe tissues exhibit higher activity respect those in leaf tissues (data not shown) and for this reason, mesocarp tissues of fruit were revealed as the eligible materials to get a move on olive protein purification methods that will preserve biological activity.

On the basis of authors records, the β-glucosidase enzyme in *Olea europaea* has been characterized by *in situ* activity studies [13], but it has not yet been purified and it is not commercially available.

Notoriously leaf and fruit tissues of olive tree (*Olea europaea* L.) are recalcitrant to the common methods for protein extraction due to the presence, at cellular level, of non protein compounds that severely interfere with the extraction in aqueous buffers. When proteins are extracted by directly homogenizing olive leaf in aqueous buffers and then precipitated by organic solvents, polyphenols and other contaminants are co-purified with the proteins, consequently the resultant brownish pellet, due to polyphenols oxidation, is hard to be dissolved [14]. These contaminants interfere also with electrophoresis separation of proteins and subsequent process of purification of class of proteins. High quality protein preparation from olive leaf was obtained by means of a non aqueous extraction that allowed good electrophoresis profiles of proteins [15]; on the other hand it caused the irreversible loss of biological activities. By contrast, extracts of leaf proteins using an aqueous sodium borate buffer (pH 9.0) showed high biological activities for the enzymes [11] but poor profiles on SDS-PAGE gels, revealing several bands with high background [16].

To our knowledge, procedures for the high purification of β-glucosidase extract from olive tissues preserving the biological activities are still not reported.

The use of membrane operations to separate complex mixtures of labile macromolecules offers an attractive alternative since they are able to preserve the biological stability.

In the last decade, there has been considerable interest in developing membrane systems for the purification of complex protein mixtures for biotechnological, food and biomedical application [17]. The reliability of using affinity-ultrafiltration [18] and diafiltration for high resolution protein fractionation has been largely demonstrated [19-29]. The configurations that have mainly been used for protein fractionation are ultrafiltration with total recycle, batch or discontinuous diafiltration, continuous single-stage ultrafiltration [27].

Traditionally, ultrafiltration has been employed for size based separation of protein mixtures where the ratio of the protein molecular mass is at least around 7-10 [28]. To achieve better purification of similarly sized biomolecules, considerable research has taken place focusing on the operating and physicochemical conditions to attain higher selectivity [20, 21, 23, 30].

Diafiltration allows removing effectively small components from the retained species by washing them out. In this process, water or buffer solution is added to the retentate during the filtration, with the membrane-permeating species being removed from the feed as this excess fluid is filtered through the membrane. The diavolume coefficient, which is equal to the total volume of the wash buffer divided by the initial feed volume, is a suitable parameter used to evaluate diafiltration process [31, 32].

A major challenge in the protein purification process is to verify the protein stability before and after each separation step; the enzyme activity has been revealed as a fine quality tester to evaluate this parameter [33]. A purification step that would reduce the initial activity to lower than 80% will not be considered suitable, since it will dramatically influence the overall costs of the process.

The aim of this work was to identify appropriate membrane operations and process conditions able to improve the purity of β-glucosidase present in fruit extracts as well as to save its catalytic properties.

To achieve this goal, the best ripening period that produced high concentration of β-glucosidase in the selected olive orchard, the preparation of fruit extracts containing β-glucosidase and their processing by diafiltration and ultrafiltration were investigated.

The diafiltration of olive fruit extracts was carried out through polyamide 50 kDa and polysulphone 30 kDa. The diafiltered permeate solutions were concentrated using 4 kDa polyethersulfone ultrafiltration membrane.

The processing by membrane technology allowed to improve the β-glucosidase purity in the fruit extracts by improving also its specific activity.

5.2 Materials and Methods

5.2.1 Plant materials

The Italian cultivar Carolea of *O. europaea*, was chosen for the experiments. During two consecutive harvest seasons (2003 and 2004) olive fruits, grown in the Calabria region, were randomly picked by hand from the established groves at the following different stages of fruit ripening: *i)* green immature fruit with woody endocarp, *ii)* green mature fruit, *iii)* green-brown fruit.

5.2.2 Preparation of β-glucosidase fruit extract

Olives at different stage of ripening were collected from a selected orchard in two consecutive harvesting seasons. Fruits (n=5) were washed with distilled water and immediately frozen in liquid N_2 and then destoned using a mortar and pestle. Typically, 1 g fresh pulp (n = 6 different sample for each sampling time) was ground in liquid N_2 using a mortar and pestle. The obtained frozen powder was further ground to a fine powder by the aid of quartz sand and then transferred on ice in 10 ml tubes and resuspended in 12.5 ml 0.1 M borate buffer, pH 9.0, 6% (w/v) PVP (poly-vinil pirrolidone), 1% (w/v) b-mercaptoethanol, 1.0 mM PMSF (phenylmethylsulfonylfluoride) according with the procedures described by Briante et al [11] The suspension was shaken gently for 1h at 4°C and centrifuged in a minifugue at 27000 g for 1h. The upper oil phase was carefully removed and the aqueous phase, representing a protein extract and the enzyme enriched phase, was filtered on paper and stored at -80 °C for further analyses.

5.2.3 β-glucosidase assay and protein content in the fruit extracts, permeate and retentate solutions

The β-glucosidase activity toward pNPGlc (*p*-D-nitrophenyl-β-D-glucopyranoside) [7-14] was evaluated in the fruit extracts and in processed solutions (permeate and retentate) at 37 °C by measuring the increase in absorbance at 405 nm of the reaction medium composed by 200 mM Na-phosphate buffer adjusted to pH 4.6. The linear coefficient to calculate the concentration of the reaction product was measured by calibration curve made with standard solutions of *p*-nitrophenol (Sigma-Aldrich) and corresponded to 14.0 $M^{-1}cm^{-1}$. The enzyme specific activity was expressed as mmoles of *p*-nitrophenol produced per minute at 25 °C per mg of proteins (mmol/min·mg).

Protein content in the various solutions was determined by Bradford spectrophotometric assay [34].

Membranes before experiments were rinsed with ultrapure water to eliminate additives used to preserve the membrane.

The variability, reported in all the figures, for β-glucosidase activity and mass protein content is due to the variability present in the different assayed fractionation.

5.2.4 Membrane equipment

A schematic draw of the membrane diafiltration equipment is shown in Fig. 5.1. Polyamide capillary membranes of 50 kDa nominal molecular weight cut-off (NMWCO) (PA 50 kDa) and polysulphone of 30 kDa (PS 30 kDa) were used. The structure of this kind of membranes is asymmetric with the selective layer on the lumen side and the sponge layer on the shell side.

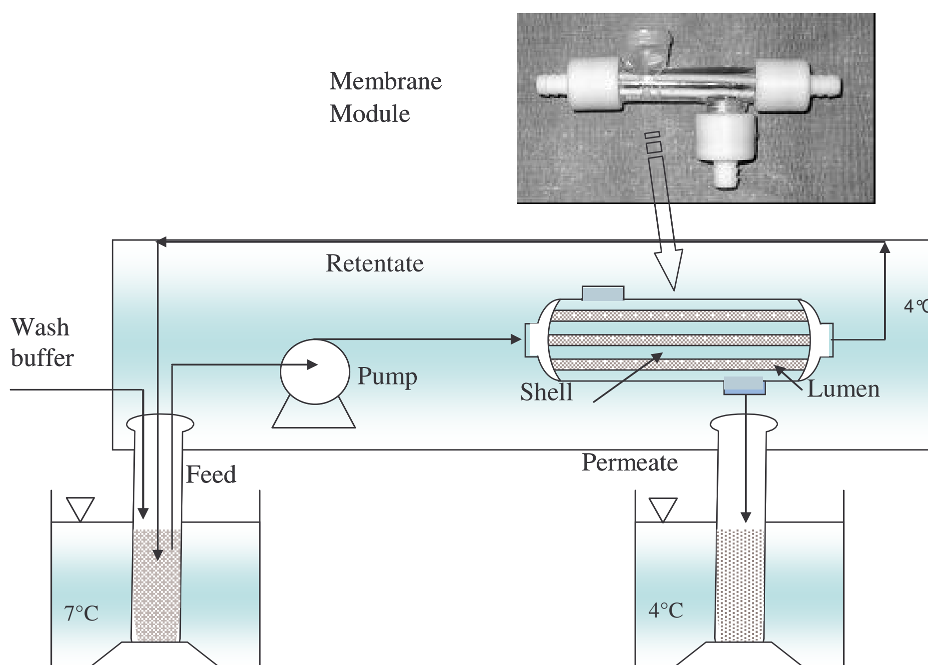


Fig. 5.1. Schematic draw of membrane diafiltration system

The lab-made membrane modules were prepared by assembling the capillary membranes inside a pirex glass cylinder.

The PA 50 kDa were assembled in modules of 1.2 cm I.D., 22 cm long, while the PS 30 kDa were assembled in modules of 1.2 cm I.D and 5.5 cm long. The internal membrane surface area was $6.9 \cdot 10^{-3}$ m^2 for PA 50 kDa and $4.60 \cdot 10^{-4}$ m^2 for PS 30 kDa. A Masterflex pump (Cole Parmer) was used to supply the feed solution to the module and to recirculate the retentate solution along the lumen circuit, during diafiltration with PA 50 kDa membranes, while an Ismatec multichannel pump (Cole-Parmer) was used during diafiltration with PS 30

kDa membrane. The experiments were carried out by mantaining the permeate solution and the membrane module at 4(±1) °C while the feed solution was mantained at 6(±1) C°. The permeate was collected from the shell side. The wash buffer used for diafiltration of protein extract from *Olea europaea* was 0.1 M borate buffer pH 9.

Experiments were carried out at flow rate of about 55 ml/min (0.09 m/sec) during diafiltration with 50 kDa PA membranes and 0.96 ml/min (0.005 m/sec) during diafiltration with 30 kDa PS membranes.

Depending on the diafiltration coefficient, the concentration of proteins permeated through the membrane and collected in the permeate can be very low. In order to be able to detect proteins in these samples by electrophoresis the permeate solution was concentrated by flat membranes made of polyethersulphone having 4 kDa NMWCO (NADIR, Germany). The permeate concentration was carried out in a dead-end cell.

5.2.5 Electrophoresis

Proteins present in the collected samples (feed, permeate and retentate) were analyzed by one-dimensional SDS-PAGE according to Laemmli [35] in two different apparatus: Bio-Rad mini-Protean II apparatus and a 10-15% PhastGel™ gradient using buffer strips. For the first procedure, Laemmli buffer system was used to cast 6% stacking and 12,5% resolving gel, except that the resolving gel contained 12,5% glycerol and the final concentration of the resolving gel buffer (Tris-HCl, pH 8,8) was 0.75M rather than original 0.0375M. After denaturation at 95°C for 3 min, proteins were resolved at 200mV.

For the second procedure, an 8/1 μl sample applicator was used (Amersham Biosciences, UK). The gel has a continuous 10 to 15% gradient gel zone with 2% crosslinking. The buffer system in PhastGel SDS Strip is composed of 0.20 M Tris-glicine, 0.20 M Tris and 0.55% SDS, pH 8.1.

Sample preparation: to final volume, 2.5% SDS (Sigma-Aldrich) and 5% β-mercaptoethanol (Sigma-Aldrich) were added and heated at 100°C and then 0.01% of bromophenol blue (Sigma-Aldrich) was added. Each sample was loaded onto separate lane of the gel containing 1 μL of sample. The gels were stained with silver and then distained with 3.7% Tris-HCl, and 1.6% sodium tiosulphate. The solution for preserving the gels contained 10% glycerol.

The gel images captured by scanner were analysed by Image Quant TL Software (Amersham Biosciences, UK), which permitted to identify band molecular weights (MW) and concentration.

5.2.6 Western blotting

Western blot analysis of feed, permeate and retentate after SDS-PAGE electrophoresis was performed using a polyclonal anti-β-glucosidase as the primary antibody, kindly supplied by Dr Y. Minami [36]. The proteins electroblotted on membrane were blocked overnight at room temperature with 3% BSA in TBST (20 mM Tris-HCl, pH 7.5; 0.8% mM NaCl; 0.1% Tween 20) and then incubated with the primary antibody for 1 h at room temperature at a dilution of 1:2000. After washing in TBST, membranes were incubated with secondary antibody (anti rabbit alkaline phosphatase, AP, conjugate) at a dilution of 1:3000 for 1h at room temperature. The detection was performed using the alkaline phosphatase detection system with the anti-biotin NBT/BCIP (4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate) reagent kit (Roche). The antibody recognized a protein band at 65 kDa molecular weight as a putative olive β-glucosidase [36]. The β-glucosidase from almond (Sigma, St Louis, USA) was used as standard purified enzyme.

5.2.7 Dot blot

The protein samples of feed, permeate and retentate samples are spotted through circular templates directly onto the nitrocellulose membrane (Hybond ECL, Amersham) and allowed to dry. Then, the membrane is incubated with 3% BSA in TBST (20 mM Tris-HCl, pH 7.5; 0.8% mM NaCl; 0.1% Tween 20) for 30 minutes. The membrane is incubated with the primary antibody (polyclonal anti-β-glucosidase) for 1 h at room temperature at a dilution of 1:2000. After washing in TBST, membrane was incubated with secondary antibody (anti rabbit alkaline phosphatase, AP, conjugate) at a dilution of 1:3000 for 1h. The detection was performed following the procedures described for western blot. The β-glucosidase from almond (Sigma, St Louis, USA) was used as standard purified enzyme.

5.3 Results and Discussion

In this section, the results of the experiments carried out to identify the maturation stage at which β-glucosidase showed the maximum activity in the olive fruit and the processing of extracts from this stage by diafiltration and nanofiltration will be presented and discussed.

5.3.1 Optimal ripening stage expressing high β-glucosidase activity in olive fruit

Results from enzyme activity assay during fruit ripening are consistent with a gradually increase of enzyme activity in green maturation phase, corresponding approximately from 60 to 180 days after anthesis (Fig. 5.2).

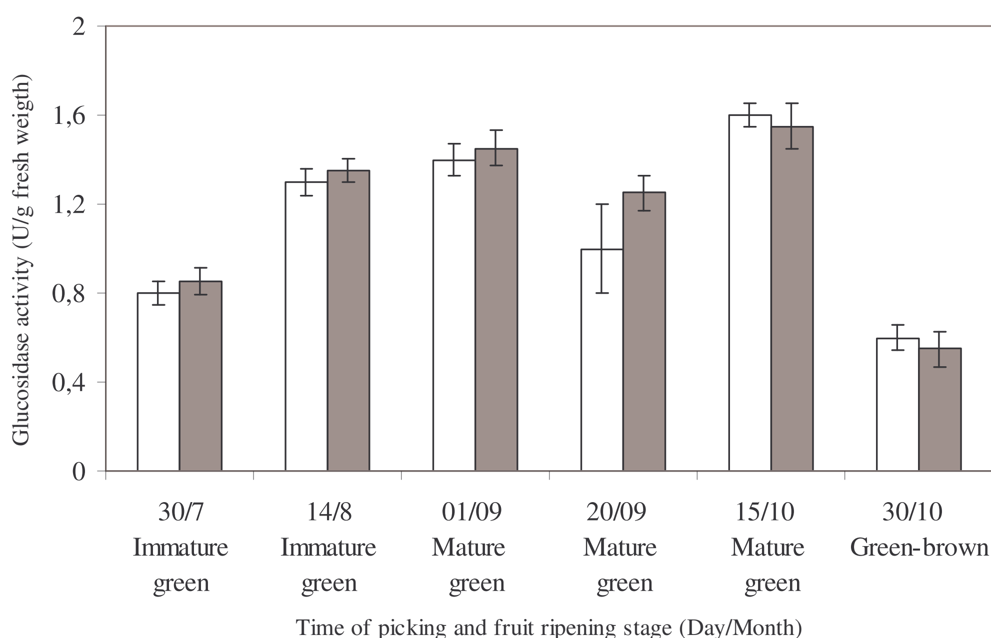


Fig 5.2. β-glucosidase activity in olive fruits extracts at different ripening stages during two different harvest seasons: 2003(☐), 2004(■). Enzyme activity was evaluated toward the synthetic substrate pNPGlc at pH 4.6

In fact, the enzyme activity found in fruit having not wooded endocarp was very low, while it increased in the pulp of stoned immature green fruits and reached the maximum at green maturation phase. Subsequently, a significant decrease of enzyme activity was detected in fruits undergone to black maturation. Considering the long sampling intervals (from July 2003 to November 2004) and consequent fruit growth differences and the variation of environmental conditions, the reproducibility of β-glucosidase activity resulted very good (Fig. 5.2). In other olive cultivars, Briante et al [11] showed an equivalent trend of β-glucosidase activity levels during ripening, suggesting that, in general, in olive the green maturation is the stage of ripening at which the enzyme expression reaches its maximum. However, we found mean activity values for each sampling time higher (e.g. 1.4 units per g of fresh weight) than those reported for the other cultivars (e.g. 0.8 units of fresh weight) [11]. Recently, evidences that these significant differences might be due to different enzyme isoforms have been reported [13].

On the basis of these results, fruit extracts from green maturation stage were produced and downstream purified by membrane processes.

5.3.2 Processing of fruit extracts by diafiltration

5.3.2.1. Diafiltration through polyamide 50 kDa membrane and permeate concentration by ultrafiltration

60 ml of initial extract solution were diafiltered through PA 50 kDa using 0.1 M borate buffer pH 9. Afterwards, the enzyme activity of the collected fractions and qualitative analysis by electrophoresis (feed, retentate and permeate) were measured versus diafiltration volume (diavolume: total wash buffer volume/initial feed volume). The amount of protein in the initial solution and in the processed fractions was determined by Bradford assay. As shown in Fig. 5.3 a, the mass in the initial solution was 22.09 (± 0.2 mg) while in the final permeate and retentate solutions was 4.78 (± 0.4 mg) and 17.96 (± 0.4 mg), respectively.

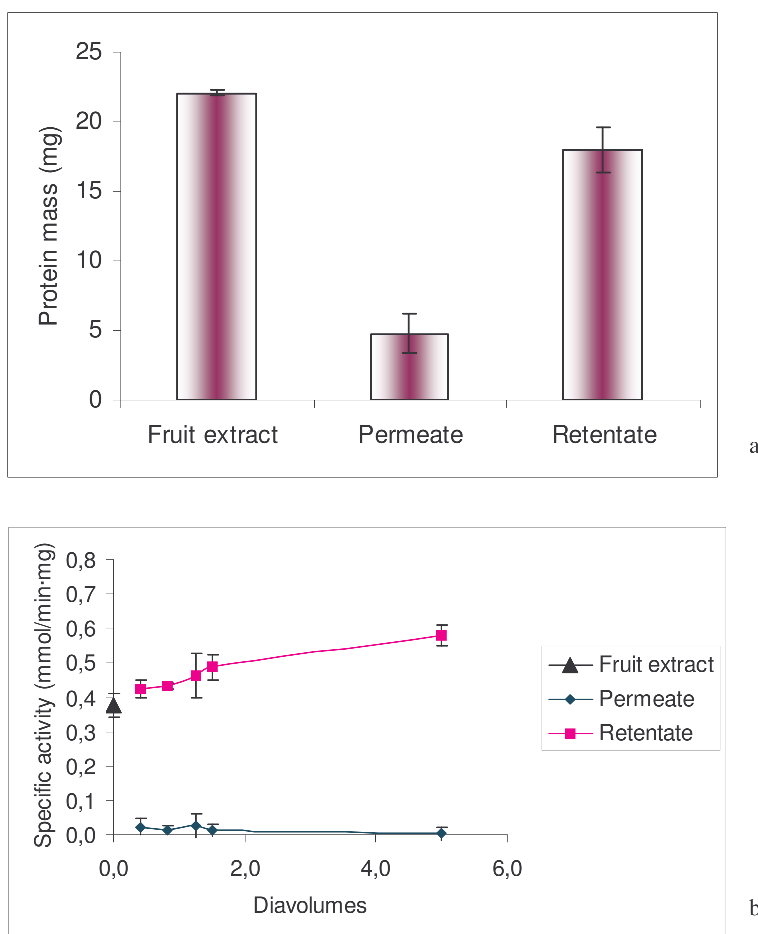


Fig 5.3. Protein mass (a) and β-glucosidase activity (b) in solutions from diafiltration through PA 50 kDa membrane of fruit extract from *Olea europaea*.

The β-glucosidase activity as a function of diavolume coefficient was also investigated. As shown in Fig. 5.3 b, the specific activity of initial solution was 0.380 (± 0.03) $mmol/min \cdot mg_{protein}$, while in the final permeate and retentate solutions were 0.003 $mmol/min \cdot mg_{protein}$ and 0.580 (± 0.020) $mmol/min \cdot mg_{protein}$, respectively. The specific activity

present in the retentate solution increased as a function of diavolume. After diafiltration of 4.5 diavolumes the specific activity in the retentate solution increased of about 52% compared to the initial one. On the other hand, the protein present in the permeate solution did not show any activity.

A qualitative analysis in the initial solutions and in the collected samples was carried out by dot blot and electrophoresis (Fig. 5.4 a and 5.4 b).

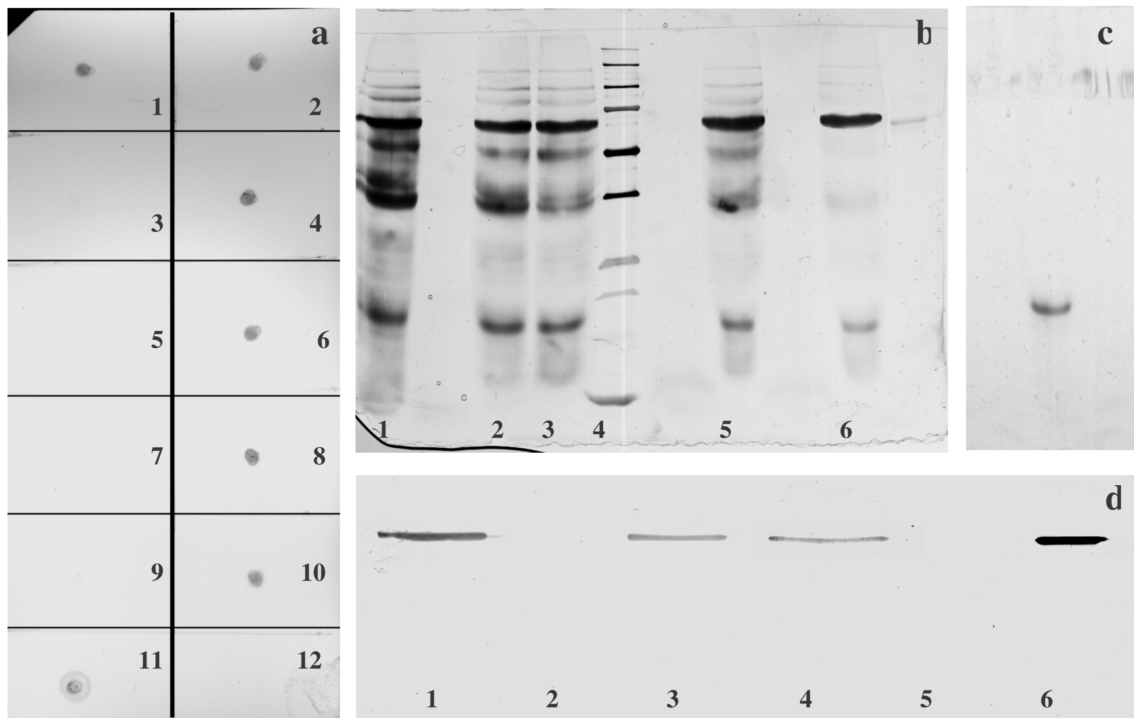


Fig 5.4. a) Dot blots performed by the antibody against β-glucosidase: 1.and 2. crude protein extract from *Olea europaea*; 3. and 4. permeate and retentate, respectively after 0.42 diavolumes; 5. and 6. permeate and retentate after 0.83 diavolumes; 7 and 8 permeate and retentate after 1.25 diavolumes; 9. and 10. permeate and retentate after 1.50 diavolumes. 11 β-glucosidase from almond; b) SDS-PAGE : lane 1. protein extract from *Olea europaea*; lane 2. retentate after 0.42 diavolumes; lane 3. retentate after 0.83 diavolumes; lane 4. marker BioRad; lane 5. retentate after 1.25 diavolumes; lane 6. retentate after 1.50 diavolumes; c) permeate after concentration by nanofiltration membrane; d) Western blots: lane 1. crude protein extract from *Olea europaea*; lane 2. permeate after 0.42 diavolumes; lane 3. retentate after 0.42 diavolumes; lane 4. retentate after 0.83 diavolumes; lane 5. permeate after 1.25 diavolumes; lane 6. retentate after 1.25 diavolumes.

In order to detect the protein in the permeate solutions, it was necessary to concentrate them. The solutions were concentrated using 4 kDa poliethersulfone ultrafiltration membranes. The suitable concentration factor was about 7, i.e. a volume of 20 ml was reduced to 3 ml. In no one permeate solution the band of the β-glucosidase (65 kDa) was present, as demonstrated by the dot blot tests carried out with a specific antibody for β-glucosidase (Fig. 5.4 a). These

results confirmed that β-glucosidase did not pass through the membrane of 50 kDa. In fact, as can be seen in Fig 5c only a protein of 20 kDa, as a major representative band, was detected in the permeate solution after concentration. The intensity of β-glucosidase (65 kDa) in the retentate solutions remained constant, in addition the profile of background became more clear during diafiltration process, (Fig 5.4 b, compare line: 1 initial solution and lanes 2, 3, 5, 6: retentate solutions after 0.42, 0.83, 1.25, 1.50 diavolumes). In all the solutions analyzed the main band was β-glucosidase as confirmed by western analysis carried out with a specific antibody for β-glucosidase (Fig. 5.4 d). The antibody recognizes a single protein band of 65 kDa. Since at least two different β-glucosidases were found in the cells of fruit tissues [13], our results seem to be consistent with the purification of one of the two isoforms. Although we have yet no evidences to identify which isoform is or whether the band corresponded to both enzymes having an equivalent molecular weight, in our view the extraction buffer used, with low ionic strength and without detergents, could be able to extract only the cytoplasmic isoform, since it could be not effective in breaking the chloroplasts and removing the isoform inside them [1].

The processed β-glucosidase extract increased the specific activity not only due to removal of non catalytic protein, but also because of removal of inhibitors. In fact, the specific activity (0.580 $mmol/min \cdot mg_{protein}$) obtained considering only the mass present in the retentate was higher than the one (0.460 $mmol/min \cdot mg_{protein}$) evaluated considering the total protein present in the retentate and permeate and it was also higher compared to the specific activity (0.380 $mmol/min \cdot mg_{protein}$) of the initial extract solution.

5.3.2.2 Diafiltration through polysulphone 30 kDa membrane

In order to identify other intermediate molecular weight fractions, diafiltration through membranes with different membrane cut-off was carried out. Preliminary water permeability tests confirmed that no significant adsorption occurred on the polysulfone membrane.

2 ml of initial extract solution were diafiltered through PS 30 kDa using borate buffer pH 9. Also in this series of experiments diavolume was 4.5. After diafiltration, quantitative analysis for measuring protein mass (by Bradford test) and catalytic activity were carried out. Also in this case, electrophoresis analysis allowed the qualitative identification of protein content. As illustrated in Fig. 5.5, the amount of protein in the fruit extract (feed) and in the retentate solution were 0.90 (± 0.30) mg and 0.84 (± 0.30) mg, respectively. The β-glucosidase specific activities related to these samples were 0.16 (± 0.03) $mmol/min \cdot mg_{protein}$ for the feed and 0.11 (± 0.02) $mmol/min \cdot mg_{protein}$ for the retentate.

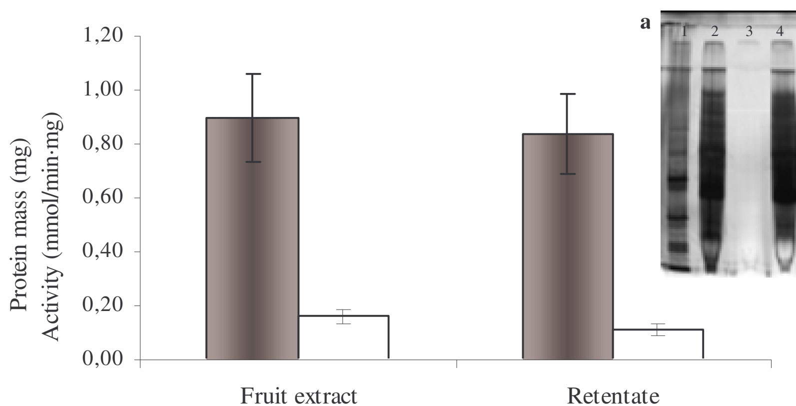


Fig 5.5. Protein mass (■) and β-glucosidase activity (□) in solutions from diafiltration through PS 30 kDa polysulphone membrane of fruit extract from *Olea europaea*. a) SDS-page assay: lane 1. marquer; lane 2 protein extract from *Olea europaea*; lane 3 permeate;

The electrophoresis tests of fruit extract, permeate and retentate (Fig 5.5a) confirmed these results; in fact, the composition of fruit extract and retentate was not changed. The background in this case remained constant and for this reason it was not possible to carry out analysis of western and dot blot.

These results indicated that, although the same diavolume was practised, the protein extract was not purified through PS 30 kDa. This was due to the fact that inhibitors have a molecular weight larger than 30 kDa; therefore the 20 kDa proteins detected in the previous experiments must be present as complexes in the native fruit extract.

5.4 Conclusions

The *Olea europaea* maturation stage, in which β-glucosidase is largely expressed in tissues, was identified in the mature green phase of ripening, according with previous findings. Fruits extract from this stage were prepared and processed by diafiltration and ultrafiltration. The process allowed to separate and to identify a major enzyme form with molecular weight of 65 kDa identified as a putative β-glucosidase by a western blot analysis and enzyme activity assay and a 20 kDa monomeric protein that in the native extract is combined to form higher molecular weight complexes. The diafiltration resulted a methodology able to guarantee the protein stability. In fact, the purification allowed to increase the specific activity of β-glucosidase from *Olea europaea* extracts during diafiltration with 50 kDa PA membranes up to 52% compared to the initial activity for the measured range of diavolume values in addition better electrophoresis profiles were obtained.

References

[1] Konno K, Hirayama C, Yasui H, Nakamura M. Enzymatic activation of oleuropein: A protein crosslinker used as a chemical defense in the privet tree. Proc. Natl. Acad. Sci. USA, 1999, 96, 9159-9164.

[2] Goupy P, Fleuriet A, Amiot MJ, Macheix JJ. Enzymatic browning, oleuropein content, and diphenol oxidase activity in olive cultivars (*Olea europaea* L.). J. Agric. Food Chem. 1991, 39, 92-95.

[3] Cianfardini G, Marsilio V, Lanza B, Pozzi N. Hydrolysis of oleuropein by *Lactobacillus plantarum* strains associated with olive fermentation. Appl. Environ. Microbiol. 1994, 60, 4142-4147.

[4] Marsilio V, Lanza B, Pozzi N. Progress in table olive debittering: degradation in vitro of oleuropein and its derivatives by *Lactobacillus plantarum*. J. Am. Oil Chem. Soc. 1996, 73, 593-597.

[5] Materassi R, Miclaus N, Pelagatti O. Hydrolysis of oleuropein in yeasts. Ann. Ist Speriment Elaiotec. 1975, 5, 53-65.

[6] Saija A, Trombetta DM, Tomaino D, Lo Cascio R, Princi P, Uccella N, Bonina F, Castelli F. In vitro evaluation of the antioxidant activity and biomembrane interaction of the plant phenols oleuropein and hydroxytyrosol. Int. J. Pharm. 1998, 166, 123-133.

[7] Esen A, β-Glucosidases. In β-Glucosidases Biochemical and Molecular Biology, American Chemical Society, Washington, 1993, 1-14.

[9] Dietz KJ, Sauter A, Wichert K, Messdaghi D, Hartung W. Extracellular b-glucosidase activity in barley involved in the hydrolysis of ABA glucose conjugate in leaves. J. Exp. Bot. 2000, 51, 937-944.

[10] Cameron RG, Manthey JA, Baker RA, Grohamann K. Purification and characterization of a β-glucosidase from Citrus sinensis var. Valencia fruit tissue. J Agric. Food Chem. 2001, 49, 4457-4462.

[11] Briante R, Patumi M, Limongelli S, Febbraio FC, Vaccaio A, Di Salle F, La Cara R, Nucci M. Changes in phenolic and enzymatic activities content during fruit ripening in two Italian cultivars of *Olea europaea* L., Plant Sci. 2002, 162, 791-798.

[12] Mazzuca S, Uccella N. β-glucosidase releasing of phytoalexin derivatives from secobiophenols as defence mechanism against pathogenic elicitors in olive drupes. Acta Hort. 2002, 586, 529-531.

[13] Mazzuca S, Spadafora A, Innocenti AM. Cell and tissue localization of b-glucosidase during the ripening of olive fruit (Olea europaea) by in situ activity assay. Plant Science,

accepted for publication.
[14] Mazzuca S. Research reports of Italian National Research Council “OEVOCAL” n° 97.00138.MZ79 project (2000).
[15] Wang W, Scali M, Vignani R, Spadafora A, Sensi E, Mazzuca S, Cresti M. Protein extraction for two-dimensional electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds. Electroph. 2003, 24, 2369–2375.
[15] Granier F. Extraction of plant proteins for two-dimensional electrophoresis. Electroph. 1988, 9, 712–718.
[16] Garcia JL, Avidan N, Troncoso A, Sarmento R, Lavee S. Possible juvenile-related proteins in olive tree tissues. Sci. Hort. 2000, 85, 271–284.
[17] Douglas BB, Zydney AL. Effect of Solution on Protein Transport Through Ultrafiltration Membranes. Biotechnol. Bioeng. 1999, 64, 27-37.
[18] Klein E. Affinity Membranes Their Chemistry and Performance in: Adsorptive Separation Processes. John Wiley and Sons, New York, 1991.
[19] Higuchi A, Mishima S, Nakagawa T. Separation of proteins by surface modified polysulphone membranes. J. Membr. Sci. 1992, 57, 175- 185.
[20] Saksena S, Zydney AL. Effect of solution pH and ionic strength on the separation of albumin from immunoglobulins. J. Membr. Sci. 1994, 43, 960- 968.
[21] van Eijndhoven RHCM, Saksena S, Zydney AL. Protein fractionation using electrostatic interaction in membrane filtration. Biotech. Bioeng. 1995, 48, 406- 414.
[22] Ehsani N, Parkkinen M, Nyström M. Fractionation of natural and model egg-white protein solution with modified and unmodified polysulphone UF membranes. J. Membr. Sci. 1997, 123 105- 119.
[23] Nyström M, Aimar P, Luque S, Kulovaara M, Metsämuuronen S. Fractionation of model proteins using their physiochemical properties. Colloid and Surf. 1998, 138, 185-205.
[24] Ghosh R, Li QY. Fractionation of BSA and lysozyme using ultrafiltration: effect of gas sparging. AIChE J 1998, 44, 61-67.
[25] Zydney AL. Protein Separation Using Membrane Filtration: New opportunities for Whey Fractionation, Int. Diary J. 1998, 8, 243- 250.
[26] Drioli E, Giorno L. Biocatalytic Membrane Reactors: Application in Biotechnology and the Pharmaceutical Industry, Taylor & Francis Publisher, London, 1999.
[27] Ghosh R. Novel cascade ultrafiltration configuration for continuous, high-resolution protein-protein fractionation: a simulation study. J. Membr. Sci. 2003, 226, 85-99.

[28] Ghosh R, Wan Y, Cui Z, Hale G. Parameter scanning ultrafiltration: rapid optimization of protein separation, Biotech. Bioeng. 2003, 81, 673- 682.

[29] Feins M, Sirkar KK. Novel internally staged ultrafiltration for protein purification, J. Membr. Sci. 2005, 248, 137-148.

[30] Feins M, Sirkar KK. Highly Selective Membranes in Protein Ultrafiltration, Biot. And Biong. 2004, 86, 603-611.

[31] Zydney AL. Engineering aspect of Microfiltration/Ultrafiltration technology, in: Microfiltration and Ultrafiltration: Principles and Application. Marcel Dekker, New York, 1996, 391-392.

[32] Lipnizki F, Boelsmand J, Madsen RF. Concepts of industrial-scale diafiltration system, Desalin. 2002, 144, 179-184.

[33] Brewer S J, Sassenfeld HM. Recovery of native protein, in: Protein purification applications, a pratical approach. Oxford University Press, Tokio, 1990, 99-111.

[34] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analis. Biochem. 1976, 72, 248-254.

[35] Laemmli UK Cleavage of structural proteins during the assembly of the head of bacteriophage T$. Nat.1970, 222, 680-685

[36] Minami Y, Takao H, Kanafuji T, Miura K, Kondo M, Hara-Nishimura I, Nishimura M, Matsubara H. β-Glucosidase in the Indigo plant: intracellular localization and tissue specific expression in leaves, Plant Cell Physiol. 1997, 38, 1069.

Chapter 6

Immunolocalization of β-glucosidase immobilized within polysulphone capillary membrane and evaluation of its activity *in situ*

Abstract

A new combined method is reported to localize the sites of enzyme immobilization and to determine its catalytic activity on a polymeric capillary membrane reactor. The useful new method resulted from the merging of the classic *in situ* enzyme activity assay and western blot technique which both results are easily detectable both at low and high magnification in light microscopy. β-glucosidases from olive fruit was selected as enzyme model because of its suitable relevance in the industrial processing of foods, in biotechnology and in pharmaceuticals and for its activity against the synthetic substrate 5-brome-4-chloro-3-indolyl-β-D-glucopyranosyde which develops an insoluble dyed product. The enzyme was physically immobilized on the 30 kDa cut-off capillary membranes and results obtained by means of a polyclonal antibody against β-glucosidase and the synthetic substrate clearly showed a coherent localization of the immobilization enzyme sites and its activity.

6.1. Introduction

Immobilized biocatalysts have widespread applications in areas like organic synthesis, pollution control and diagnostics. Enzymes can be immobilized on different supports like polymeric beads, gels and membranes. Hybrid systems using immobilized enzymes, such as biocatalytic membrane reactors are well described in the open literature.

Membranes possess the distinct advantage of very high surface area for enhanced enzyme loading and can sustain convective flow mode. Immobilization eliminates the need to separate an enzyme from the product solution and allows these expensive compounds to be reused. In addition, the thermal and storage stability of an enzyme may be increased as a result of

immobilization (Butterfield 1996; Cao 2005). These advantages are in addition to those that enzymes possess over conventional catalysts such as high efficiency and regio/stereospecificity (Lye 1996). Common immobilization techniques may be grouped in two classes: physical immobilization and chemical immobilization. Physical technique include the adsorption (Pamula 2005, Rojas-Melagarejo 2004, Marron-Brignone 1996) of an enzyme onto a carrier surface and the entrapment (Shukla 2004) of an enzyme within a support. Chemical techniques (Shukla 2005) involve the formation of a chemical (e.g. covalent) bond between the enzyme and the support. While the physical immobilization often help to preserve the properties of an enzyme and facilitate regeneration, covalent attachment generally contributes a greater degree of immobilization stability (Taylor 1991). The amount of biocatalyst loaded, its distribution and activity through the support and its lifetime are very important parameters to properly orientate the development of such system. Usually the amount of immobilized protein is known by mass balance between the initial solution (feed) and the solutions after immobilization process (e.g retentate and permeate) (Drioli, 1999). The catalytic properties are then measured as observed reaction rate on the basis of the reaction product in the bulk solution as a function of time. The use of fluorescence labelling of proteins was used (Crespo 1999) to monitor protein fractionation by ultrafiltration and to control membrane fouling. Sousa et al. (2001) evaluated the distribution along hollow fibres membranes of the amount of enzyme immobilized on microporous nylon membrane. The procedure was based on measuring the protein content in membranes pieces sectioned after enzyme immobilization, along the module length, i.e. initial, middle and end of the membrane module, by Lowry assay. Liu et al (2005) proposed a method for the localization of active peroxidase immobilized on microfiltration flat membranes by the use of 3,3'-Diaminobenzidine tertahydrochloride (DAB). It can form an insoluble brown polymeric non-droplet precipitate which is strongly osmium-philic when oxidized in presence of H_2O_2. After the reaction with DAB, the membranes pieces, embedded in Epon resin, were cutted by ultra-microtome and observed by electron microscopy.

In the present work a new combined method revealing the sites where the enzyme is immobilized within the thickness of ultrafiltration polysulphone membranes and the assessment of its activity *in situ* has been developed.

This method is based on the merged procedures of *in situ* assay developed for histochemistry detection of glucohydrolase activity and the western blotting for immunodetection of target proteins on nitrocellulose membranes (Neal Burnette, 1981; Polak and Van Noorden S., 1986). The histochemical detection of glycohydrolase enzymes was developed and

extensively applied in the *in situ* detection of the expressions of GUS reported gene constructs in the transformed tissues (Jefferson et al, 1987; Bauer et al, 1993; Schmitz et al., 1990). Based on the same procedural conditions the enzyme family 1 of β-D-glucoside glucohydrolase, E.C. 3.2.1.21, specifically hydrolyzed the synthetic substrate (5-brome-4-chloro-3-indolyl-β-D-glucopyranosyde), it is routinely applied as marker of endoplasmic reticulum and useful as bacterial differentiation (Horwith, 1964; Amigorena et al., 1994; Romano, et al., 2003). In fact following the hydrolysis, the XGlc develops a blue dyed product that precipitates in the site of the reaction; consequently it is possible to visualized the localization of β-glucosidases in the cells by means the light microscope observation still at low magnifications. Actually, no evidences exist at our knowledge of the performances of the assay on enzyme loaded on the artificial moieties as polymeric membranes, except for its analogous synthetic substrate used for the glucohydrolases detection on acrylamide gels (Esen, 1993). On the other hand, besides no evidences exist on the classic western blot analysis applied on capillary polysulphone membrane loaded with the target proteins, practically, no significative challenges are expected respect to the nitrocellulose membrane performances toward the effectiveness on the epitope-antibody recognition. For this purpose the native β-glucosidase from olive fruit (*Olea europea)* was chosen as enzyme to immobilize onto the membrane because the well known catalytic activity towards the synthetic substrate able to develop the insoluble dyed products that precipitates on the sites of reaction. In addition the β-glucosidase was selected among other enzymes because of its suitable target for protein engineering to address the food processing and quality enhancement. (Günata 2003; Fenwicket al 1983), biomass conversion in biotechnology (Beguin, P. 1990), as well as substrate processing in pharmaceutical (LW Wattenberg 1971). In particular, olive β-glucosidase activity is linked to many processes such as the defense against pests and pathogens (Konno et al, 1999), the loss of bitter taste of ripens fruit pulp (Ryan et al, 1999; Brenes et al 1992) and influence foodstuff quality resulting from industrial processing of olive fruits (Morello et al 2004; Marsilio et al. 1996; Cianfardini et al 1994). Besides, the products of enzymatic hydrolysis of its natural substrate oleuropein are well known as pharmacologically active molecules (Bisignano et al, 2001).

6.2. Materials and methods

6.2.1. Enzyme extraction

Olive green fruits showing high β-glucosidase activity (1.5 ± 0.4 U/mg of proteins) were washed with distilled water and immediately frozen in liquid N_2 and then destoned using a

mortar and pestle. Typically, 1 g fresh pulp (n = 6 different sample for each sampling time) was ground in liquid N_2 using a mortar and pestle. The obtained frozen powder was further ground to a fine powder by the aid of quartz sand and then transferred on ice in 10 ml tubes and resuspended in 12.5 ml 0.1 M borate buffer, pH 9.0, 6% (w/v) PVP (poly-vinil pyrrolidone), 1% (w/v) β-mercaptoethanol, 1.0 mM PMSF (phenylmethylsulfonylfluoride) according to Briante et al. (2002). The suspension was shaken gently for 1h at 4°C and centrifuged in a minifugue at 27000 g for 1h. The upper oil phase was carefully removed and the aqueous phase, representing a protein extract and the enzyme enriched phase, was filtered on paper and stored at -80 °C. Western blot analysis of enzyme extract after SDS-PAGE electrophoresis was performed using a 1:2000 polyclonal anti-β-glucosidase as the primary antibody, kindly supplied by Dr Y. Minami (1997). The secondary antibody was a 1:3000 anti rabbit AP conjugate antibody. The detection was made by the alkaline phosphatase detection system with the anti-biotin NBT/BCIP (4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate) reagent kit (Roche). The antibody recognized a protein band at 65 kDa molecular weight as a putative olive β-glucosidase (Fig. 6.1).

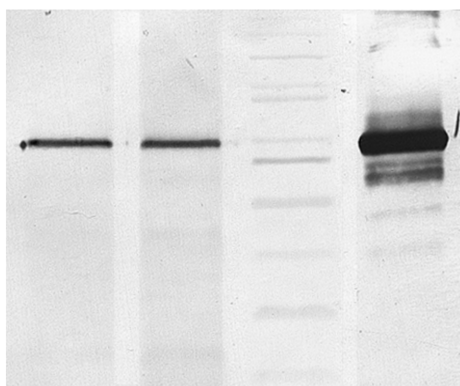


Fig 6.1. Western blot of olive tissues extracts after SDS-PAGE in which the polyclonal anti-β-glucosidase antibody recognized a protein band at 65 kDa as a putative olive β-glucosidase (lanes 1 and 2). Lane 3) 200-14 kDa markers. Lane 4) β-glucosidase from almomd (Sigma, St Louis, USA) was used as standard purified enzyme.

The β-glucosidase from almond (Sigma, St Louis, USA) was used as standard purified enzyme.

6.2.2. Structure of polysuphone membrane and module assembling

For enzyme immobilization, commercial polysulphone capillary membranes with nominal molecular weight cut-off (NMWCO) of 30 kDa, having outer diameter of 1.05 mm, were

used. The membranes were kindly provided by Romicon, Inc.. The structure of this kind of membranes is asymmetric with the selective layer on the lumen side and the sponge layer on the shell side. The lab-made membrane modules were prepared by assembling three capillary membranes inside a pirex glass cylinder of 1.2 cm I.D., 5.5 cm long. The external membrane surface area was $4.25 \cdot 10^{-4}$ m^2. Before permeability tests, the membranes were first washed with ultra pure water to remove the water-soluble residues. After this step immobilization procedure with fruit extract was carried out.

6.2.3. Immobilization of β-glucosidase from olive fruit by cross-flow ultrafiltration

5 ml of fruit extract solution was recirculated along the shell side at a flow rate of 1.45 ml/min, axial velocity of 0.028 m/s and a transmembrane pressure of 0.10 bar. The enzyme solution permeated from shell to lumen. In this way the β-glucosidase, present in the extract (65 kDa) entered the spongy layer but could not pass through the thin layer (cut-off 30 kDa). After the cross-flow ultrafiltration was completed the membrane was rinsed in order to remove the amount of the enzyme reversibly adsorbed on the membrane. Two rinsing cycles of 5 minutes each with 0.1 M borate buffer pH 9 were carried out, with a flow rate of 2.38 ml/min, axial velocity of 0.046 m/s and a transmembrane pressure of 0.15 bar.

Same immobilization procedures with pure β-glucosidase from almond were carried out.

An Ismatec peristaltic pump (Cole-Parmer) was used to supply the feed solution to the module and to recirculate the retentate stream along the shell circuit. The experiments were carried out by maintaining the feed solution and the permeate at 4°C and the membrane module at room temperature. The fractions collected after immobilization procedure were 0.8 ml of permeate and 4.2 ml of retentate. Protein content in the various solutions was determined by Bradford spectrophotometric assay (Bradford, 1976). The amount of immobilized protein was calculated by mass balance:

$$\text{Mass}_{\text{of immobilized protein}} = \text{Mass}_{\text{feed}} - (\text{Mass}_{\text{retentate}} + \text{Mass}_{\text{permeate}} + \text{Mass}_{\text{washing solution}})$$

6.2.4. In situ assay activity of β-glucosidase

The three fibers loaded with the enzyme were disassembled from the modules and freshly sectioned at 4°C by using a vibrotome (Leica), along fibers length (initial, middle and end of the fibers) obtaining the serial sections each of 80 μm in thickness. Longitudinal and cross sections were immediately placed in the minivials containing 1 ml of detection buffer, adapted for β-glucosidase from Jefferson (1), containing 60 μM of the synthetic substarte (5-

brome-4-chloro-3-indolyl-β-D-glucopyranosyde, Sigma, St Louis), 50 mM phosphate buffer pH 6.5, 1.5 mM potassium ferricianide, 1.5 mM potassium ferrocianide, 10 mM EDTA pH 8.0. After enzymatic hydrolysis by immobilized β-glucosidase, the synthetic substrate develops an insoluble blue product that precipitates on the site of reaction thus localizing the active enzyme in the membrane. For each sampling three membrane sections, initial, middle and end of the fibers, were placed in the vials contained the detection buffer without the synthetic substrate as the controls. Sections were incubated in a wet chamber at 37 °C in the dark for 30 min, 1 h, 2 h and 3 h. The reaction was stopped in cold phosphate buffer and the sections were mounted with glycerol on slides for optical microscopy and digitalized by Leitz Dialux EB microscope equipped with a CCD camera. Image analysis on three sections for each membrane replicate (n= 9) was performed by QWin™ Image System Software (Leica).

6.2.5. Immunoblotting on β-glucosidase- loaded membrane

Subsequent to the *in situ* detection of β-glucosidase activity the enzyme-loaded membrane sections were processed using a modified protocol drawn from conventional western blot analyses (Burnette 1981). The membrane sections were embedded in a drop of blocking buffer (pH 7.6, 0.025% Tween, 5% normal serum). After washes the primary polyclonal anti β-glucosidase antibody was added in blocking buffer for 1 h at room temperature. The antibody dilutions were from 1: 2000 to 1:5000. Secondary anti-rabbit antibody gold conjugated (Inalco spa) at the dilution 1: 6000 in TBST with 2% normal serum was added and incubated for 1 h at room temperature. For light microscopy analyses, secondary antibodies linkages were visualized on membrane by silver enhancing kit (Sigma, St Paolo, MO, USA). As control, for each antibody dilution, membranes were processed excluding the incubation with primary anti β-glucosidase antibody. Same membrane sections were processed for immunoblotting, immediately after cuttings, without to perform the *in situ* activity in order to ascertain the alterations on enzyme distribution within the membrane due to the assay conditions.

6.3. Results and Discussion

6.3.1. Physical immobilization of β-glucosidase from olive fruit

The immobilization of β-glucosidase was achieved by cross-flow ultrafiltration from shell to lumen through a polysulphone membranes which initial pure water permeability was 263.62 (±43.81) l/hm^{2}bar. During the protein extract filtration the permeability dropped to 9.41(±

42.81) l/hm^2bar. This severe decrease was due to the high concentration polarization caused by the low axial velocity 0.028 m/s used in order to preserve the biological activity of the enzyme.

As described in the materials and methods section, the total protein content of the collected samples after ultrafiltration were determinated by Bradford assay.

The amount of immobilized protein for fruit extract was about 0.32 (± 0.05) mg and was calculated as mass balance from values shown in Table 6.1. During immobilization with pure β-glucosidase from almond the amount of immobilized enzyme was 0.03 (± 0.02) mg.

Table 6.1. Example of samples collected from protein extract ultrafiltration

Sample	Concentration (mg/ml)	Volume (ml)	Mass (mg)
Feed	0.400 (± 0.060)	5.000	2.000 (± 0.300)
Permeate	0.020 (± 0.003)	0.800	0.024 (± 0.004)
Retentate	0.380 (± 0.057)	4.200	1.630 (± 0.240)
Washing solution 1	0.090 (± 0.013)	4.000	0.024 (± 0.004)
Washing solution 2	0.000	4.000	0.000

In the case of fruit extract immobilization, the mass immobilized doesn't represent the effective β-glucosidase content but the total protein content present in the membrane. This information does not indicate where the enzyme is located and how it is distributed through the membrane thickness and along the module length.

6.3.2. In situ assay activity of β-glucosidase

The vibrotome cut is useful for certain difficult materials such as very soft biological specimens or in the case of investigation of some specific activities or molecules that are inactivated and destroyed by fixatives or embedding procedures. The sections obtained can be relatively thick (50 - 100 μm), however this method is suitable for light microscopy analyses. The enzyme loaded membrane appeared to be easily sectioned and handily manipulated by means of this technique, even though it was not possible to obtain good cutting accuracy of longitudinal and cross-sections of less than 70 μm in thickness. Cutting took place in the physiological buffer thus minimizing damage to loaded enzyme and maintaining the bath temperature of 4°C. Also, the operating vibrating blade was kept constant with a vibration frequency of 2. As shown in Fig. 6.2 (a, b) cross and longitudinal vibrotome membrane sections, observed in light stereoscopy, preserved a good structure.

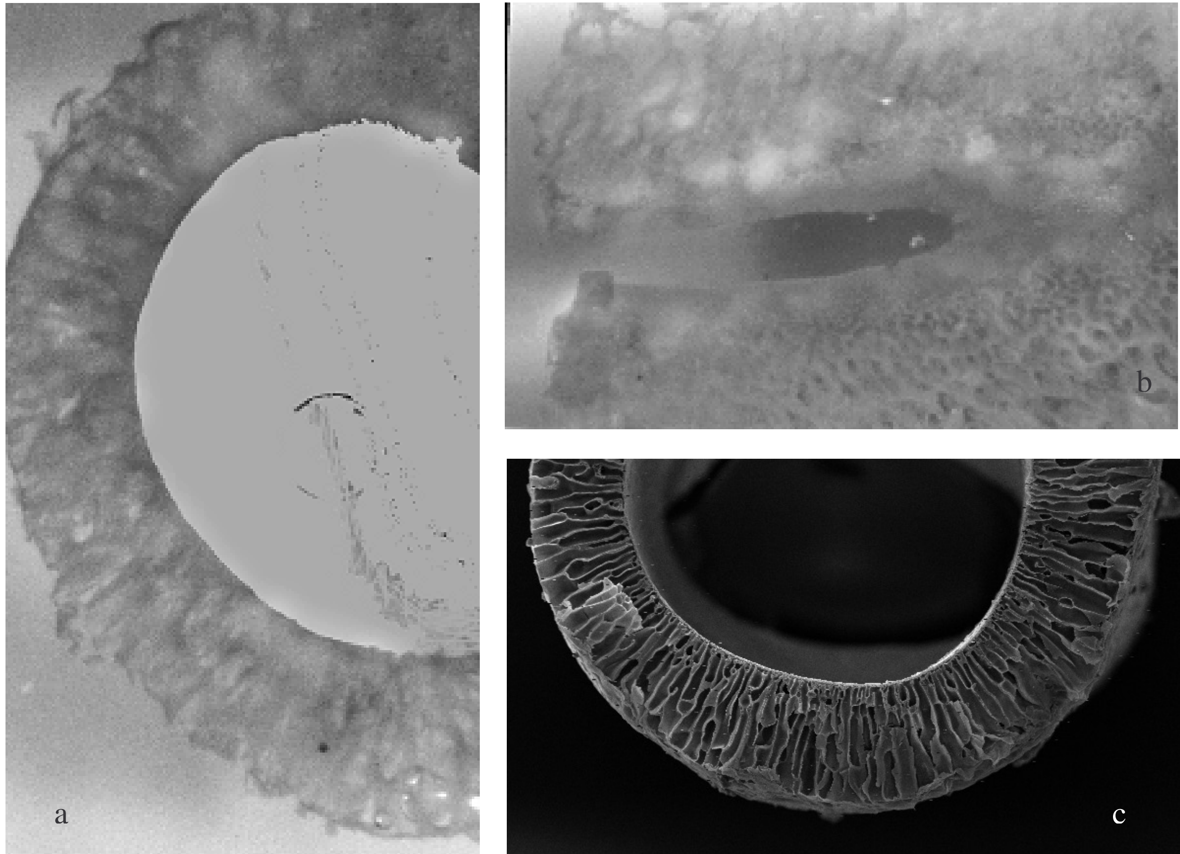


Fig 6.2. Cross (a) 40 X and longitudinal (b) 60X membrane sections 80 μm in thickness obtained by the vibrotome and observed in light stereoscopy (40 X). c) Scanning electron microscopic picture of cross section of 30 kDa polysulphone capillary membrane 30 X.

The typical structure of this kind of membranes, with the selective layer on the lumen side and the sponge layer on the shell side resulted to be not damaged or deformed in the texture by cutting. The asymmetric membrane structure is illustrated in the part c of Fig. 6.2, which shows a scanning electron microscopic pictures of cross section of 30 kDa polysulphone capillary membrane.

After enzyme immobilization, the activity in situ was measured by using the reaction illustrated in Fig. 6.3.

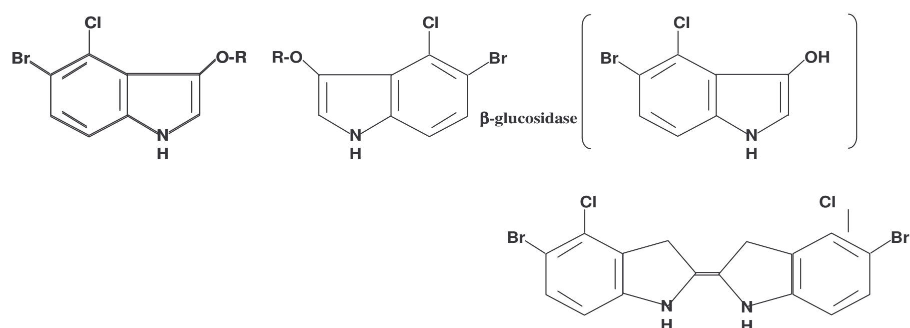


Fig 6.3. Scheme of the reaction used for the *in situ* enzyme activity assay

In the Fig 6.4, cross sections of fibers at middle level of the module are reported. Similar results were obtained along the fiber length at the beginning and end of the module.

From microscope pictures in Fig 6.4 (d, e, f) it can clearly be seen the uniform spreading of the insoluble blue colour of the product obtained by enzymatic hydrolysis of the synthetic substrate which can be supposed the representative site of active β-glucosidase immobilized in the membrane. In order to minimize the product diffusion within the membrane, the detection buffer for β-glucosidase activity assay contained an higher concentration of ferricianide and ferrocianide (up to 20%) respect to those in the biology standard assay (Jefferson, 1987). The results showed the enzyme was distributed through the membrane thickness along the all module length. Therefore the cross-flow ultrafiltration in the used operating conditions cab be considered a useful method to immobilize enzyme through the overall membrane.

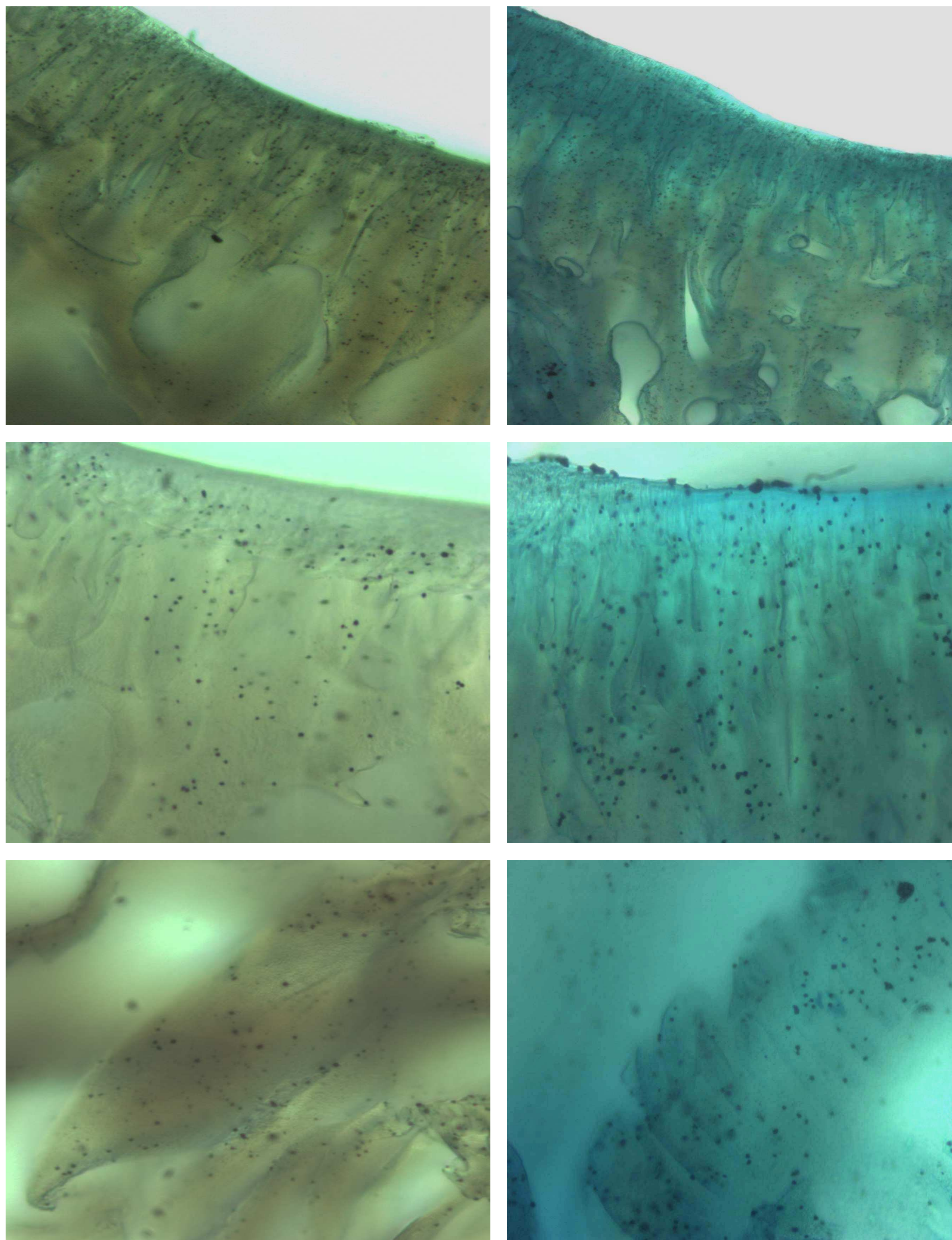


Fig 6.4. Microphotographs of β-glucosidase-loaded membrane sections (a)(b) and (c) after the immunolocalization with the antibody against β-glucosidase; (d)(e) and (f) after the combined methods of immunolocalization and *in situ* activity assay. (a) 100 X of sample on the lumen side; (b)(c) 400 X of samples on the lumen side and shell side, respectively. (d) 100 X of sample on the lumen side; (e)(f) 400 X of samples on the lumen side and shell side, respectively.

6.3.3. Immunoblotting on β-glucosidase-loaded membrane

A preliminary analysis of polyclonal antibody against β-glucosidase is performed in the western blot carried out on the electrophoreses proteins under denaturant conditions. The antibody recognizes both β-glucosidase from olive extract and commercial purified almond β-glucosidase, as a single protein band of 65 kDa (Fig. 6.1), as previously reported in materials and methods section. The anti-β-glucosidase is specifically against the chloroplastic β-glucosidase from leaves of *Polygonum tinctorium* (Minami et al, 1997) and, in this case, demonstrated to have high specificity for β-glucosidase from different plant sources. On these bases the immunoblotting on β-glucosidase loaded polysulphone membrane is performed using a protocol drawn from traditional western blot for immunocalization on nitrocellulose membrane (Burnette 1981). The major modifications made consist in the decrease of detergent concentration (up to 30%) of the blotting solutions and in the employ of floating membrane sections on the drops of solutions which minimized the amount of antibodies required. Immunoblottings from β-glucosidase loaded membranes were performed prior Fig 6.4 (a, b, c) and after (d, e, f) the *in situ* assay. The microscope observations showed that the antibody recognized the immobilized β-glucosidase since the black spots are localized inside the membrane. The spots size and number, in fact, develop from the amount of the linkages of gold-conjugated secondary antibodies which are silver enhanced to allow the observation of the linkage in light microscopy (Polak and Van Noorden 1986). A control test was performed by carrying out the immunoblotting procedure on membranes containing no enzyme, in this case no spots were observed.

The spots appear to be distributed through the thickness, either within the selective layer on the lumen side than in the sponge layer on the shell side. Seldom, on the membrane lumen there are larger spots, which means that enzyme may accumulate more there since it is retained by the thin layer. The *in situ* assay appears to do not influence the spots distribution along the membrane surface and do not affect the efficiency of epitope-antibody recognition in the membrane section as indicated by the equal amount of spots number and spot size between the two treatments (Fig 6.4, a *vs* d). As the enzyme localization and its activity *in situ* are considered the results from a combined method of the immunoblotting *in situ* assay, they clearly showed a correlation between the catalytic activity and the sites of enzyme immobilization, confirming that the colour distribution is not due to product diffusion inside the membrane, but corresponded to the presence of the immobilized active enzyme in the site of reaction. These results pointed that the physical immobilization can provide high

membrane surface for the enzyme to be immobilized and shows that the biocatalysts can be distributed through all the membrane thickness. The combined method was also verified on membrane loaded with pure β-glucosidase from almond as standard, results are reported in Fig 6.5.

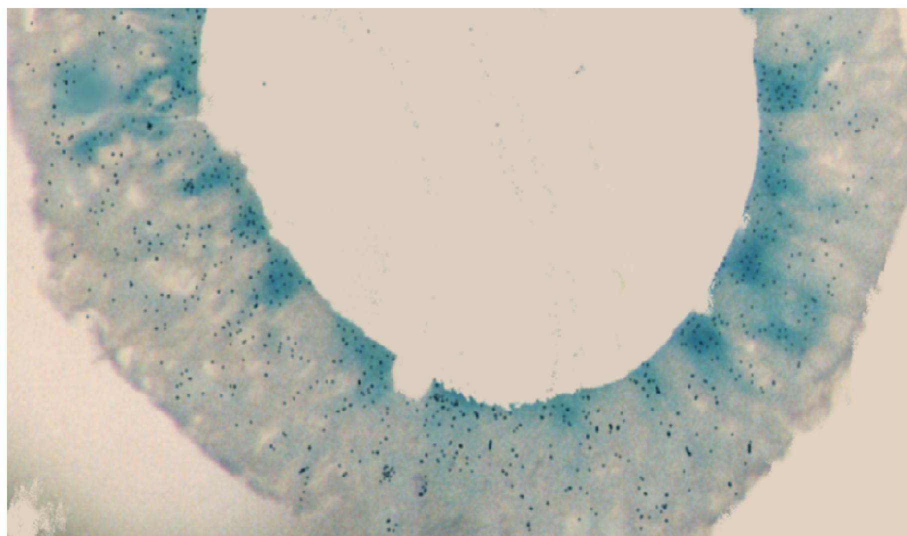


Fig 6.5 Cross section of pure β-glucosdidase from almond loaded-membrane, after combined methods of immunolocalization and *in situ* activity assay (40 X).

Also in this case the results showed that the enzyme was immobilized through all the membrane thickness with major presence on the thin layer.

6.4. Conclusion

A new combined method merged from the classical *in situ* detection of enzyme activity and western blot analysis was applied for the first time to the capillary asymmetric polysulphone membrane reactor to determine simultaneously the enzyme spatial distribution through the membrane thickness and along the membrane module and its activity after the immobilization through the observation in the light microscopy both at low and high magnifications. Results revealed a coherent localization of enzyme and its insoluble product of hydrolysis through the all membrane which evidenced that physical entrapment by cross-flow ultrafiltration allowed the enzyme macromolecules to penetrate through the membrane module preserving the catalytic activity. The described approach can be useful to clarify crucial informations for the optimisation of the enzyme immobilization techniques for estimating the enzyme content over a non pure protein starting solution for measuring

intrinsic kinetic properties of immobilized enzymes, for the modelling of membrane reactors and their development at large scale.

References

[1] D. Allan Butterfield, Biofunctional Membranes, Plenum, New York, NY, 1996.

[2] L. Cao, Immobilized enzymes: science or art? Curr. Opin. Chem. Biol. 9 (2005) 217.

[3] G.J. Lye, O.P. Pavlou, M. Rosjidi, D.C. Stuckey, Immobilization of Candida cylindracea Lipase on Colloidal Liquid Aphrons (CLAs) and Development of a continuous CLA-Membrane Reactor, Biotech. Bioeng. 51 (1996) 69.

[4] S.P. Shukla, K. Modi, P.K. Gosh, S. Devi, Immobilization of horsedarish peroxidase by entrapment in natural polysaccharide, J. Appl. Polym. Sci. 91 (2004) 2063.

[5] S.P. Shukla, S. Devi, Covalent coupling of peroxidase to a copolymer of acrylamide (AAm)-2-hydroxyethyl methacrylate (HEMA) and its use in phenol oxidation, Prog. Biochem. 40 (2005) 147.

[6] E. Pamula, P.G. Rouxhet, Influence of surface properties of carbon fibres on the adsorption of catalase, Carbon 43 (2005) 1432.

[7] F. Rojas-Melgarejo, J.N. Rodriguez-Lopez, F. Garcia-Canovas, P.A. Garcia-Ruiz, Immobilization of horsedarish peroxidase on cinnamic carbohydrate ester, Prog. Biochem. 39 (2004) 1455.

[8] L. Marron-Brignone, R.M. Morélis, P.R. Coulet, Immobilization through adsorption of lusiferase on Langmir-Blodgett film. Influence of the hydrophilicity or hydrophobicity of the surface on the enzyme kinetic behaviour, Langmuir 12 (1996) 5674.

[9] R.F. Taylor, Protein immobilization: Fundamentals and Applications, Marcel Dekker, Inc., New York, NY, 1991.

[10] E. Drioli, L. Giorno, Application in Biotechnology and the Pharmaceutical Industry: Biocatalytic Membrane Reactors, Taylor & Francis, London, 1999.

[11] J.P.S.G. Crespo, M. Trotin, D. Hough, J.A. Howell, Use of fluorescence labelling to monitor protein fractionation by ultrafiltration under controlled permeate flux, J. Membr. Sci. 155 (1999) 209.

[12] H.A. Sousa, C. Rodrigues, E. Klein, C.A.M. Afonso, J.G. Crespo, Immobilisation of pig liver esterase in hollow fibre membranes, Enzyme Microb. Technol. 29 (2001) 625.

[13] Z.M. Liu, J. Dubremez, V. Richard, Q. Yang, Z.K. Xu, P. Seta, Useful method for the spatial localization determination of enzyme (peroxidase) distribution on microfiltration membrane, J. Membr. Sci. 267 (2005) 2.

[14] W. Neal Burnette, Western Blotting: Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A, Anal. Biochem. 112 (1981)195.

[15] J. Polak, S. Van Noorden. "Immunocytochemistry. Modern methods and applications." J. Wright and Sons, Bristol, 1986.
[16] R.A. Jefferson, T.A. Kavanagh, M.W. Bevan, GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants, EMBO J., 6 (1987) 3901.
[17] R. Bauer, F. Paltauf, S.D. Kohlwein, Fuctional expression of bacterial β-glucuronidase and its use as a reporter system in the yeast *Yarrowia lipolytic,* Yeast 9 (1993) 71.
[18] U.K Schmitz, D.M. Lonsdale, R. Jefferson, Application of the β-glucuronidase gene fusion system to saccharomyces cerevisiae, Curr. Genet., 17 (1990) 261.
[19] J.P. Horwith, A histochemical substrate for β-glucosidase, J Med. Chem 7 (1964) 574.
[20] S. Amigorena, J.R. Drake, P. Webster I. Mellman**,** Transient accumulation of new class II MHC molecules in a novel endocytic compartment in B lymphocytes, Nature 369 (1994) 113.
[21] I. Romano, A. Giordano, L. Lama, B. Nicolaus, A. Gambacorta, *Planococcus rifietensis* sp. nov, isolated from algal mat collected from a sulfurous spring in Campania (Italy), Syst Appl Microbiol. 26 (2003) 357.
[22] A. Esen, β-Glucosidases, in β-Glucosidases: Biochemical and Molecular Biology, ed. Esen A., American Chemical Society, Washington, 1993, pp 1.
[23 Z. Günata, Flavor enhancement in fruit juices and derived beverages by exogenous glycosidases and consequences of the use of enzyme preparations, in J. R. Whitaker, A. G. J. Voragen, and D. Wong (eds.) Handbook of Food Enzymology. Marcel Dekker, Inc., Portland, 2003.
[24] G.R. Fenwick, R.K. Heaney, W.J. Mullin. Glucosinolates and their breakdown products in food and food plants. CRC Crit. Rev. Food Sci. Nutr. 18(1983) 123.
[25] P. Beguin,. Molecular biology of cellulose degradation, Annu. Rev. Microbiol. 44 (1990) 219.
[26] L.W. Wattenberg. Studies on polycyclic hydrocarbon hydroxylases of the intestine possibly related to cancer. Effect of diet on benzopyrene hydroxylase activity, Cancer 28 (1971) 99.
[27] K. Konno, C. Hirayama, H.Yasui and M.Nakamura Enzymatic activation of oleuropein: A protein crosslinker used as a chemical defense in the privet tree, Proc. Natl. Acad. Sci. USA, 96 (1999) 9159-9164
[28] D. Ryan, K. Robards, S. Lavee, Changes in phenolic content of olive during maturation, Int J. Food Sci. Technol. 34 (1999) 265.

[29] M. Brenes Balbuena, P. Garcia Garcia, A. Garrido Fernandez, Phenolic compounds related to the black color formed during the processing of ripe olives, J. Agric. Food Chem. 40 (1992) 1192.

[30] J.R. Morello, M.J. Moltiva, M.J. Tovar, M.P. Romero, Changes in commercial olive oil (cv Arbequina) during sorage with special emphasis on phenolic fraction, Food Chem. 85 (2004) 357.

[31] V. Marsilio, B. Lanza, N. Pozzi, Progress in table olive debittering: degradation in vitro of oleuropein and its derivatives by *Lactobacillus plantarum*, J. Am. Oil Chem. Soc., 73, (1996) 593.

[32] G. Cianfardini, V. Marsilio, B. Lanza, N. Pozzi, Hydrolysis of oleuropein by *Lactobacillus plantarum* strains associated with olive fermentation, Appl. Environ. Microbiol. 60 (1994) 4142.

[33] G. Bisignano, M.G. Laganà, D. Trombetta, S. Arena, In vitro antibacterial activity of some aliphatic aldehydes from Olea europea L. L., FEMS Microbiology Letters 198 (2001) 9.

[34] R. Briante, M. Patumi, S. Limongelli, F. C. Febbraio, A. Vaccaio, F. Di Salle, R. La Cara, M. Nucci, Changes in phenolic and enzymatic activities content during fruit ripening in two Italian cultivars of *Olea europaea* L., Plant Sci. 162 (2002) 791.

[35] Y. Minami, H. Takao, T. Kanafuji, K. Miura, M. Kondo, I. Hara-Nishimura, M. Nishimura, H. Matsubara, β-Glucosidase in the Indigo plant: intracellular localization and tissue specific expression in leaves, Plant Cell Physiol. 38 (1997) 1069.

[36] M. M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Analis. Biochem. 72 (1976) 248.

Chapter 7

Kinetic study of a biocatalytic membrane reactor containing immobilized β-glucosidase for the hydrolysis of oleuropein

Abstract

Kinetic parameters of β-glucosidase from almond immobilized in a continuous membrane reactor have been studied and compared with free enzyme used in a stirred tank reactor. The enzyme has been immobilized by cross-flow ultrafiltration in asymmetric capillary membranes made of polysulphone having nominal molecular weight cut-off of 30 kDa. The hydrolysis of oleuropein into aglycon and glucose has been used as a reaction system to demonstrate the efficiency of biocatalytic membrane reactors using immobilized β-glucosidase. The membrane allowed to support the enzyme in a defined microenvironment, the microporous matrix, as well as to control substrate feeding to and product removal from the reaction environment in a single unit operation.

Results showed that kinetic parameters of immobilized enzyme are the same as for the free enzyme, meaning that the enzyme immobilized within the membrane did not undergo decrease of activity while maintaining higher stability compared to the free enzyme. This confirms that the common observation of inverse relationship between activity decrease and stability increase for immobilized enzymes is not a general rule. In particular, the catalytic activity of immobilized enzyme can be optimized by the use of high throughput microstructured membrane systems.

7.1. Introduction

Phenolic compounds are a complex but important group of naturally occurring compounds in plant. Although the main phenolic compounds in olive fruit are secoiridoid derivates, leaves also contain phenyl acids, phenyl alcohols, and flavonoids. The increasing interest towards these compounds is due to the fact that they have pharmacological properties, are natural

antioxidants, and inhibit Gram positive micro-organisms involved in fermentation of olive fruit [1].

Oleuropein is a heterosidic ester of elenoic acid and 3,4 -dihydroxy-phenylethanol (hydroxytytrosol) [2] that produces bitterness in not ripened olives. This is the major phenolic compound in the olive fruit and leaves, and it is completely absent from olive oil, because of its high water solubility [3]. Relevant hypotensive dose-dependent effects and protection against induced arrhythmias, shown in rats, by various extract of *Olea europaea* were correlated with oleuropein content in bud and leaf preparation. It is also the high concentration of oleuropein and its hydrolysis products that confers to *Olea europaea* part to its resistance to microbial attack [4]. Other studies demonstrated that the hydrolysis products of oleuropein, thanks to β-glucosidase action, have a much more marked inhibitory effect than oleuropein. In particular, the aglycon, produced by oleuropein hydrolysis, is well known as pharmacologically active molecule. In fact, it is described for its potential application as an antimicrobial agent in some fairly common diseases of olive trees [5] and also for its strong active antioxidant property [6][7][8][9][10].

β-glucosidases, the enzymes responsible for the oleuropein hydrolysis, belong to the group of hydrolases widely existing in various sources, such as fungi, bacteria, plant and animal tissue [11]. Almost all these kind of enzymes in plant showed best pH stability values between 5-7 [12][13][14].

The broad optimum pH and temperature range makes this enzyme particularly useful for application in biotechnology processes. The β-glucosidase from different source has been studied in various systems, as free or attached to different supports [12][14][15][16][17][18][19]. Enzymatic hydrolysis of oleuropein, in a NMR tube (a thin glass walled tube used to contain samples in nuclear magnetic resonance spectroscopy) by a β-glucosidase at 25°C giving two diastereoisomeric aglycons was described [20].

The β-glucosidase activity was also tested on biological *in vivo* systems like *lactobacillus plantarum* [21] and using recombinant enzymes from hyperthermophilic archeon.

The enzymatic hydrolysis of oleuropein, extracted from olive plant, by free β-glucosidase from almond was studied by Capasso et al [22].

Homogeneous recombinant β-glycosidase from hyperthermophilic archaeon *Sulfolobus solfactaricus* expressed in *E. Coli* (EcS*β*Gly), was immobilized on chitosan support and used to produce hydroxytyrosol from commercially available oleuropein [1]. In addition the antioxidant properties of the main products obtained were purified and characterized [23] until a production of hydroxytyrosol of 91-94% in weight was achieved [24].

As recently described by Guiso et al. [25] the first step of oleuropein hydrolysis in the reaction catalyzed by β-glucosidase from almond, gives two products: glucose and aglycon.

The aglycon is interesting due to the strong antioxidant activity. Oleuropein is abundant in nature, easily available and considered as a waste material [22]. The production of aglicon from oleuropein has glucose as co-product. The development of bioprocesses based on oleuropein-β-glucosidase system for the production of aglycon and glucose represents a sustainable production strategy able to valorise wastes while approaching to zero discharge.

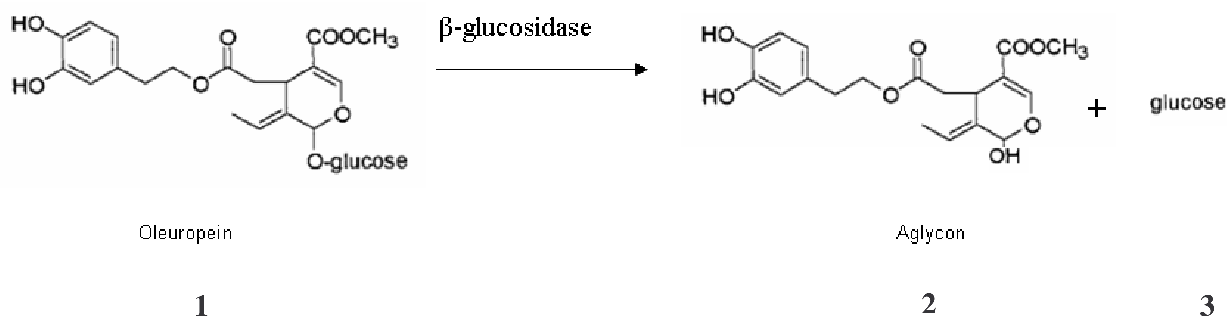
In line with this strategy, biocatalytic membrane reactors are able to integrate bioconversions with selective membrane separations leading to continuous clean, safe and low energy consumption production systems [26][27][28][29][30][31][32][33]. Enzyme immobilization in biocatalytic membrane reactors for production of high added value components in pharmaceutical, food and biotechnology has been widely studied. Examples of systems developed at industrial production level have been recently reviewed [28]. Although their potentialities have not been fulfilled yet, the current needs and challenges in satisfying the increasing consumer demand of safe goods and the limited resources availability will force towards selective and efficient technologies such as biocatalytic membrane reactors.

In this work, the development of a continuous reactor system based on β-glucosidase-loaded membrane for the conversion of commercial oleuropein into glucose and aglycon was studied. In particular, the work aimed at studying the kinetic properties of β-glucosidase immobilized in a continuous membrane reactor and its comparison with free enzyme used in a conventional batch reactor. β-glucosidase from almond was used as enzymatic model system to proof the viability of the proposed continuous reaction process for oleuropein hydrolysis compounds production.

7.2. Materials and Methods

The reaction equation of the oleuropein (**1**) hydrolysis to aglycon (**2**) and glucose (**3**) catalyzed by β-glucosidase from almond is illustrated in Fig. 7.1.

The β-glucosidase from almond (chromatographically purified lyophilized powder, Cod G4511, one unit will liberate 1.0 μmole of glucose from salicin per min at pH 5.0 at 37 °C) and glucose were obtained from Sigma Aldrich, oleuropein from Extrasynthese (France). Acetic acid (Carlo Erba) and sodium acetate (Sigma Aldrich) were used to prepare 50 mM acetate buffer pH 6.5.



1 2 3

Fig. 7.1. Scheme of the first step of the reaction catalyzed from β-glucosidase from almond having oleuropein as substrate

Acetonitrile and *o*-phosphoric acid for HPLC mobile phase preparation were purchased from Carlo Erba and Sigma Aldrich, respectively.

7.2.1. Analytical methods

Protein concentration was measured by Bicinchoninic Acid Kit (BCA) (Sigma Aldrich).

A method adapted from Ranalli et al. [34] was used to measure oleuropein by HPLC. A reverse silica LiChoCART Superspher RP8 column, 250-4 mm, 5 µm (Merck) was used. The mobile phase was a mixture of acetonotrile/water (21:79) acidified with *o*-phosphoric acid (up to pH 3). The flow rate and pressure were 0.8 ml/min and 210 (± 4) bar, respectively. The volume of sample injection was 20 µl. The oleuropein was detected at 280 nm wavelength.

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.

Aglycon was calculated as equivalent moles of glucose on the basis of the reaction stoichiometry. For precise thorough kinetic measurement, it is mandatory to instantaneously stop the reaction in the collected samples.

For reactions carried out in the biocatalytic membrane reactor, the enzyme is immobilized in the membrane and samples are collected as permeate solution, therefore enzyme is not present in these samples, the reaction cannot occur anymore and they do not require any further treatment for enzyme deactivation and separation from other components.

Samples withdrawn from reactions carried out with free enzyme need fast deactivation of the enzyme, in order to stop the reaction, and its separation from the other components, in order to avoid interferences with HPLC and spectrophotometric analyses.

Among the various chemical physical methods to deactivate enzyme, the thermal deactivation was identified as the most suitable method due to the possibility to avoid the use of chemicals that may cause interference with HPLC analyses. The sample pre-treatment method was

optimized by identifying the temperature and minimum incubation time necessary to deactivate the enzyme. Enzyme solution samples were immersed in a bath at 95°C for 30, 60, 120, 180 s and after that the enzyme activity was measured. After the denaturizing step, the enzyme was separated from the other compounds present in the sample solution by ultrafiltration. Polysulphone (PS) (Romicon, Germany), polyethersulphone (PES) (PBI international) and regenerated cellulose (PBI international) of 30 kDa molecular weight cut-off (MWCO) were used. The most suitable membrane was selected on the basis of its inertness towards the other reaction components commercially available and tested to monitor the reaction rate, i.e. oleuropein and glucose (aglycon is not commercially available yet). Therefore, to verify that no adsorption of these molecules on polymeric membranes was occurring during sample treatment, ultrafiltration tests with standard solutions through the different polymers were carried out. Adsorption was calculated by mass balance between initial and permeate solutions.

7.2.2. Equipments

Hydrolysis reaction catalyzed by free β-glucosidase was carried out in a stirred tank reactor (STR), which is characterized by time variant conditions (Fig. 7.2 a). The reaction catalyzed by immobilized enzyme was performed in a continuous biocatalytic membrane reactor (BMR), which is characterized by time invariant conditions at the steady state (Fig. 7.2 b).

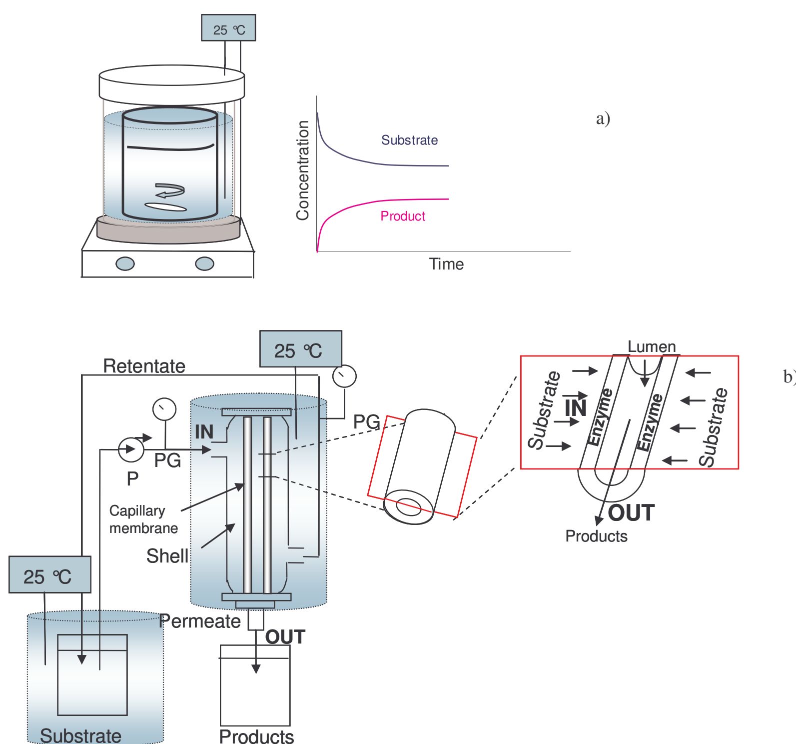


Fig. 7.2. Experimental reactors set-up. a) Stirred tank reactor and corresponding concentration profile of substrate and product. b) Experimental apparatus used to carry out reaction in the continuous biocatalytic membrane reactor and corresponding concentration profile of substrate and product. (P: peristaltic pump, PG: pressure gauges)

7.2.2.1. Stirred tank reactor (STR) setup

The reaction took place in a tank with 50 ml total volume of reaction mixture, 45 ml of substrate solution plus 5 ml (0.035 mg/ml). The enzyme solution was prepared dissolving 3.5 mg in 10 ml of buffer solution at pH 6.5, to obtain a final concentration of 0.35 mg/ml. 5 ml of this enzyme solution, were then added to 45 ml of oleuropein solution to obtain a final concentration of 0.035 mg/ml. The concentration obtained was also confirmed by BCA test. The reaction tank was kept at 25 °C in a thermostatic bath. Complete mixing was achieved by using a magnetic stirrer set at 50 rpm. Samples were taken as function of time to measure the amount of oleuropein converted and corresponding glucose produced.

7.2.2.2. Biocatalytic membrane reactor (BMR) setup

The equipment used to carry out the hydrolysis reaction is illustrated in Fig. 7.2b. It consists of a membrane module made of a pyrex cylinder as shell containing four polysulphone capillary membranes of 30 kDa nominal molecular weight cut-off (NMWCO) (PS 30 kDa) and internal/external diameter of 1.08/1.75 mm. The membranes have asymmetric structure with the selective layer on the lumen side and the sponge layer on the shell side. The membrane module effective length was 10.5 (± 0.5) cm and the internal/external membrane surface area was $1.46 \cdot 10^{-3}/2.36 \cdot 10^{-3}$ ($\pm 4 \cdot 10^{-4}$) m^2. The membrane void volume was 0.61 (± 0.02) cm^3. This represents the membrane reactor volume. The membrane void volume was calculated as difference between the volume of the membrane cylinder (0.69 cm^3) (which includes polymer and void volume) and the volume occupied by the polymer (0.08 cm^3). The volume of the membrane cylinder was calculated on the basis of the fiber internal/external diameter and length. The volume of the polymer was calculated from the polymer density (1.24 g/ml) and the mass of the dried fibers.

The system is constituted also by a tank containing the oleuropein solution, a peristaltic pump (P) to feed the reagent solution to the biocatalytic membrane reactor, pressure gauges (PG) to measure inlet and outlet pressure and a graduated cylinder to collect permeate solution containing reaction product.

7.2.3. Enzyme immobilization by cross-flow ultrafiltration

After module preparation, the pure water permeability of virgin native membranes was measured from Darcy’s equation

$$J = L_p \Delta P \quad (1)$$

as the linear coefficient (L_p) between flux and applied transmembrane pressure as driving force.

This was done in order to state initial membrane performance. The β-glucosidase was entrapped within the membrane asymmetric sponge layer by cross-flow ultrafiltration from shell to lumen [26]. In fact, the 65 kDa MW enzyme macromolecules could enter the macrovoids of the sponge layer but could not pass through the 30 kDa cut-off thin layer. During the immobilization process, permeate flux was measured as a function of time and when fouling due to enzyme entrapment reached steady state the process was stopped. The membrane system was rinsed to remove all reversibly adsorbed protein and pure water permeability was measured again to evaluate hydraulic resistance promoted by immobilized enzyme. The membrane was rinsed with buffer using a pressure of 0.1 bar and an axial velocity of 1.03 m/s.

The overall characteristics of flux reduction during ultrafiltration can be described by [35]:

$$J = \frac{TMP}{\mu R} \quad (2)$$

where J ($l \cdot h^{-1} \cdot m^{-2}$) is the flux, TMP is the transmembrane pressure (bar), μ is the viscosity (Pa·s) and R (m^{-1}) is the hydraulic resistance. From Eqs.1 and 2 the hydraulic resistance is:

$$R = \frac{1}{\mu L_p} \quad (3)$$

The total hydraulic resistance is also given by:

$$R_{TOT} = R_m + R_{rev} + R_{irr} \quad (4)$$

Where R_m is the resistance due to the membrane, calculated considering the pure water permeability of virgin membranes; R_{rev} is the resistance due to reversible fouling; R_{irr} is the resistance due to irreversible fouling calculated considering the pure water permeability after washing steps. The sum between R_{rev} and R_{irr} gives the resistance due to the overall fouling R_f.

The amount of immobilized enzyme was calculated by mass balance and its contribution to hydraulic resistance was evaluated by Eqs. 3 and 4 as R_{irr}.

7.2.4 Reaction operation mode in the biocatalytic membrane reactor

After the enzyme was immobilized within the membrane and the system rinsed to remove the reversible fouling, the shell circuit was recirculated with oleuropein solution, in buffer at pH 6.5, which permeated through the membrane thickness (where the enzyme was loaded) (Fig 7.2b) with an applied transmembrane pressure of 0.1 bar, an axial velocity of 0.35 m/sec, and a permeate flow rate of 0.30 (±0.05) ml/min. A thermostatic bath was used to keep the temperature constant at 25°C. As oleuropein (**1**) (540.23 Da) passed through the enzyme-loaded membrane it was converted into the corresponding reaction products, aglycon (**2**) and glucose (**3**).

In order to confirm that changes in substrate and product concentration were only due to the biocatalyst action and no interference with the membrane materials was occurring, blank experiments were carried out, where no enzyme or deactivated enzyme was present in the membrane.

7.2.5 Evaluation of reaction rate, kinetic parameters, and enzyme stability

The initial reaction rate (V_0) for stirred tank reactor and biocatalytic membrane reactor obtained as a function of different substrate concentrations [S] were used to fit Michaelis-Menten equation thus obtaining estimates of K_m and V_{max}. ore accurate graphical evaluation of K_m and V_{max} was obtained by the Lineweaver-Burk equation [36]:

$$\frac{1}{V_0} = \frac{K_m}{V_{\max}} \cdot \frac{1}{S} + \frac{1}{V_{\max}} \tag{5}$$

The value of the kinetic constant k_{+2} was also evaluated using the following equation:

$$V_{\max} = k_{+2}[E]_{tot} \tag{6}$$

Where $[E]_{tot}$ is the total concentration of enzyme solution used in the reaction.

Equations for calculation of initial reaction rate (V_0) in the used reactor systems were derived from balance equation:

$$\text{IN - OUT + PROD = ACC} \tag{7}$$

$$(FC)_{IN} - (FC)_{OUT} + (v_r V) = \frac{dVC}{dt} \tag{8}$$

Where, *F* is the flow rate (volume/time), *C* is the concentration (mass/volume); v_r is the volumetric reaction rate (mass/time volume); *V* is the reaction volume; *t* is the time.

For the batch stirred tank reactor the flow term is zero and production is equal to accumulation:

$$v_r = \frac{dC}{dt} \tag{9}$$

Therefore, the reaction rate is calculated as time variant concentration (C) from the linear section of the curve C *vs* t (Fig. 2a).

For the biocatalytic membrane reactor, the reaction takes place at the asymmetric porous membrane level, through which IN and OUT flow terms occur mainly by convective flow. At steady state, each membrane micropore works as a continuous stirred microreactor. Therefore, the overall membrane matrix forms a high throughput reactor, whose volume is the membrane void volume which works as a continuous stirred tank reactor (CSTR).

For a steady state CSTR, the term ACC = 0:

$$(FC)_{IN} - (FC)_{OUT} + (v_r V) = 0 \tag{10}$$

The reaction rate is then derived as:

$$v_r = \frac{F(C_f - C_p)}{V} \tag{11}$$

In this equation v_r is the reaction rate ($mmol/cm^3 \cdot min$), F is the permeate flow rate (cm^3/min), V is the reactor volume (cm^3). The reactor volume is represented by the membrane void volume.

Another important parameter in membrane bioreactor is the residence time as it influences the conversion degree. High permeate flow rate means low residence time of substrate solution within the pores. The residence time (τ) was obtained from the following equation:

$$\tau = \frac{V}{F} \tag{12}$$

Where V is the membrane reactor volume (cm^3) and F is the volumetric flow rate (cm^3/s)

Enzyme stability is also a crucial parameter. Pre-screening tests were carried out to verify the stability of immobilized enzyme in order to make sure that kinetic tests were not affected by stability problems. Tests were performed as follows: the reaction was carried out through the enzyme loaded-membrane for 2.5 hours, using 2 mM oleuropein concentration. The system was then rinsed and a new reaction was carried out with a different substrate concentration. Afterwards the test with the initial substrate concentration was carried out to verify that the enzyme kept constant performance. The reactor was preserved at -80 °C overnight. The

subsequent days tests were repeated as mentioned before for a total of about 30 hours operation. The reactor was then preserved at -80 °C and after two months the activity was tested again.

7.3. Results and Discussion

Initially, the sample pre-treatment procedure was standardized, then the kinetic performance of β-glucosidase from almond immobilized in the UF-capillary membrane reactor was studied and compared with the one of free enzyme. Results are reported in the following sections.

7.3.1 Development of sample pre-treatment procedure

Experiments to set up the appropriate sample pre-treatment procedure for reaction rate evaluation as a function of time have been performed. In particular, the work focused on the identification of conditions for instantaneously stopping the reaction rate by deactivating the enzyme in samples withdrawn from stirred tank reactor and for separating the deactivated enzyme from other reaction components (e.g. oleuropein, glucose and aglycon). Results showed that 30 s of sample treatment at 95 °C were enough to denaturize the enzyme and that the high temperature did not cause any interference on the analysis of oleuropein and glucose. In fact, analytical tests of standard samples before and after treatment at high temperature, confirmed that concentration of both components remained unchanged. Aglycon was not tested as it is not commercially available.

The denaturized enzyme was then separated from the sample solution by ultrafiltration. Membranes with 30 kDa cut-off were suitable to completely remove the enzyme. Tests were performed in order to select a membrane material inert towards the reaction components, i.e. no adsorption of both substrate and product was occurring. Results are summarized in Table 7.1, which shows the values of oleuropein and glucose concentration before and after filtration through 30 kDa PES, PS and RC membrane. The amount of oleuropein after the filtration with PES membrane decreased of about 9% respect to the initial solution, due to absorption phenomena of oleuropein to the polymer.

Table 7.1. Concentration of oleuropein and glucose before and after the ultrafiltration through polyethersulphone (PES), regenerated cellulose (RC), and polysulphone (PS) membranes of 30 kDa MWCO

	PES	RC	PS
Oleuropein initial solution (mg/ml)	1.35 (± 0.02)	1.35 (± 0.05)	1.35 (± 0.03)
Oleuropein after filtration (mg/ml)	1.23 (± 0.02)	1.35 (± 0.05)	1.35 (± 0.03)
Oleuropein adsorbed to the membrane (%)	9	______	______
Glucose initial solution (mg/ml)	0.23 (± 0.02)	0.23 (± 0.02)	0.23 (± 0.02)
Glucose after filtration (mg/ml)	0.23 (± 0.02)	0.23 (± 0.02)	0.23 (± 0.02)
Glucose adsorbed to the membrane (%)	______	______	______

The mass adsorbed was calculated as mass balance between feed and permeate solution. Different behaviour was observed when the oleuropein was filtered through PS and RC membranes, where the concentration of oleuropein remained unchanged between feed and permeate solution. The glucose concentration remained the same after filtration through the three types of membranes. Therefore, both PS and RC 30 kDa membranes were suitable for the sample treatment. In subsequent experiments RC membranes have been used.

The procedure to pre-treat samples withdrawn from STR was then identified as follows: 1) stopping of the reaction by heating sample at 95 °C under agitation for 30 s; 2) cooling down the solution by immersing test tube in ice for few min; 3) separation of deactivated enzyme by ultrafiltration through RC membranes; 4) collect permeate solution for further HPLC and spectrophotometric analyses.

It is worth noting that the removal of enzyme by ultrafiltration, without first deactivating it by thermal treatment, did not result a suitable method as it did not perform a fast and reproducible stopping time of the reaction. For samples withdrawn from the continuous biocatalytic membrane reactor, this procedure was not necessary, as samples are collected as permeate from PS capillary membrane through which the reaction is occurring and which simultaneously support the enzyme and remove unconverted substrate and obtained products.

7.3.2 Reaction kinetics with β-glucosidase free in the stirred tank reactor

Experiments with free β-glucosidase from almond were carried out in order to compare the performance of native enzyme with the immobilized one, at the same operating conditions (temperature, pH, buffer concentration, and substrate). Experiments were carried out using 2,

2.5, 5, 7.5, 10, 15, 20 mM of oleuropein at pH 6.5, temperature of 25°C and enzyme concentration of 0.035 mg/ml. Fig. 7.3 illustrates an example (5mM) of the behaviour of oleuropein and glucose concentration as function of time. Both substrate decrease and product formation were measured in order to verify that results were congruent with the reaction stoichiometry (Fig. 7.1). As evidenced in the upper part of the Fig. 7.3, the moles of oleuropein disappeared were equivalent to the moles of glucose and then of aglycon produced. From this type of graph V_0 was calculated as the tangent to the curve in the linear range when the conversion is less than 5%, so that product concentration was negligible compared to the substrate concentration.

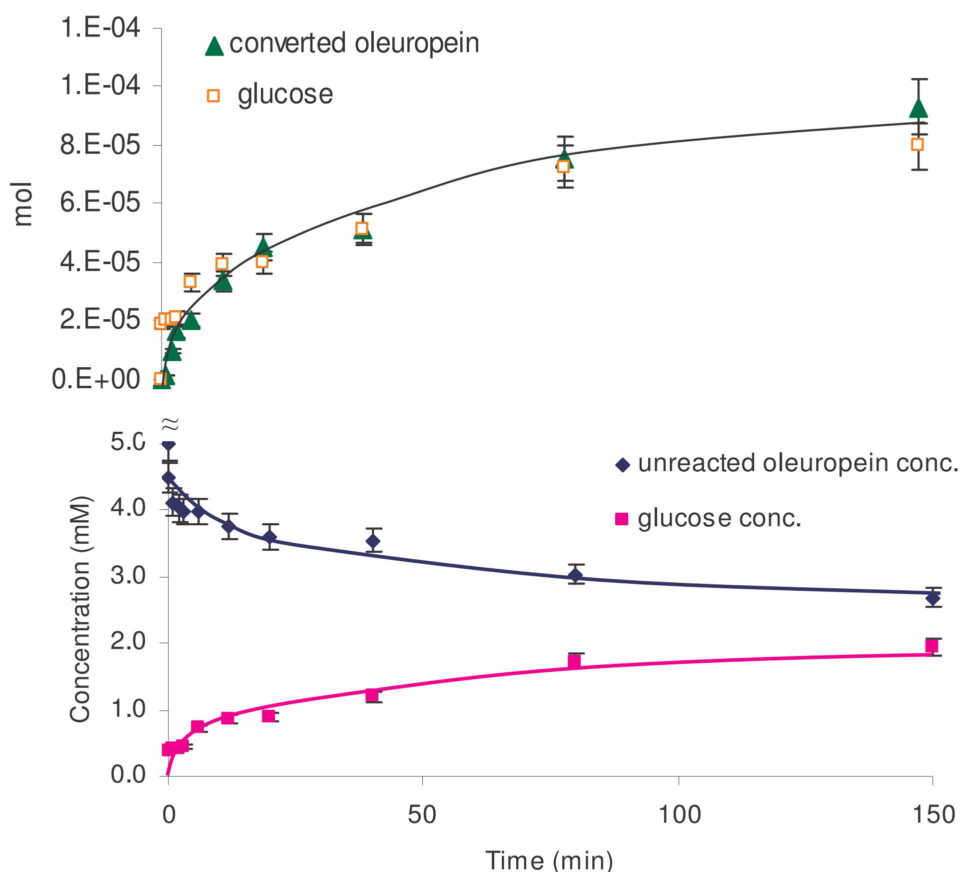


Fig. 7.3. Behaviour of unreacted oleuropein (◆) and glucose concentration (■) as function of time in the stirred tank reactor and relative correspondence to the reaction stoichiometry between mol of converted oleuropein (▲) and mol of glucose produced (□).

An overall conversion degree of 30 (± 3) % was reached after 9000 s (2.5 h) of residence time and the initial reaction rate was about $1.80 \cdot 10^{-2}$ (± 0.001) $mmol/cm^3$ h for the mentioned example. K_m and V_{max} were calculated from the Lineweaver-Burk graph illustrated in Fig. 7.4.

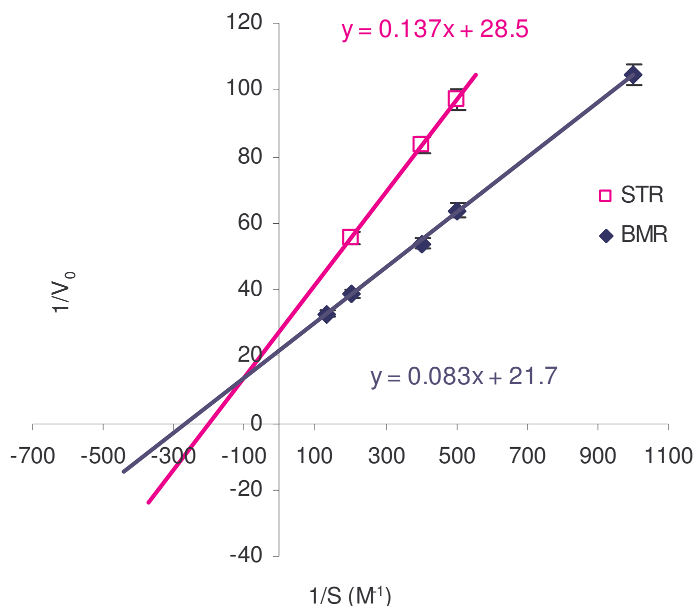


Fig. 7.4. Lineweaver-Burk plot for the enzyme free in the STR and for the enzyme immobilized in the BMR

Results show that the K_m value was comparable with the one from Capasso et al. (1997) [22], who used the oleuropein as substrate, even thought experiments were carried out at different pH and temperature

conditions.

Table 7.2. Kinetic parameters of ß-glucosidase from almond obtained in the present work and comparison with literature data

Enzyme status	Enzyme support	Substrate	K_m (mM)	V_{max} [a]	k_{+2} (s^{-1})	k_{+2}/K_m ($mM^{-1} \cdot s^{-1}$)	pH	T (°C)
Free	-	Oleuropein	4.80 (± $1.1 \cdot 10^{-1}$)	$3.5 \cdot 10^{-1}$ (± $4 \cdot 10^{-3}$) ($M \cdot h^{-1}$) $1.9 \cdot 10^{-2}$ (±$9.5 \cdot 10^{-4}$) (U/mg)[b]	18 (± $9 \cdot 10^{-1}$)	3.75	6.5	25
Immobilized (entrapped)	Polysulphone capillary membranes	Oleuropein	3.80 (± $1.4 \cdot 10^{-1}$)	$4.6 \cdot 10^{-2}$ (±$2 \cdot 10^{-3}$) ($M \cdot h^{-1}$)	18[c]	4.73	6.5	25
Free	-	Oleuropein	4.66	$3.6 \cdot 10^{-3}$ (U/mg)	-	-	5.5	37

[a] Unit is indicated per each value as authors used different units.
[b] We strongly recommend the use of "molar concentration/time" as reaction rate unit. However, since the unit U/mg (or $mmol \cdot min^{-1} \cdot mg^{-1}$) is commonly used, we also report the V_{max} according to this unit. This V_{max} was calculated from Lineweaver-Burk plot using V_0 expressed in terms of U/mg.
[c] Assumed equivalent to the one of the free enzyme on the basis of similar K_m and V_{max} values.

The higher value for V_{max} obtained in the present work compared to [22] might be due to a higher purity of the enzyme used.

The value of k_{+2}, calculated according to equation 6, is also reported in Table 7.2. The total amount of enzyme used in the STR was $5.38 \cdot 10^{-7}$ mmol/cm^3.

7.3.3. Reaction kinetics with β-glucosidase immobilized in the biocatalytic membrane reactor

Initially, blank experiments were carried out to verify that oleuropein can freely pass through the membrane without interfering phenomena and that the presence of immobilized enzyme on the membrane did not alter its transport through it. As reported in Fig. 7.5a the oleuropein concentration did not change during its passage through both the PS virgin membrane and PS membrane loaded with deactivated enzyme. In fact, the concentration in the feed (IN) and permeate (OUT) solutions were the same. This indicated that changes in oleuropein concentration in the working membrane reactor can be fully attributed to enzymatic hydrolysis.

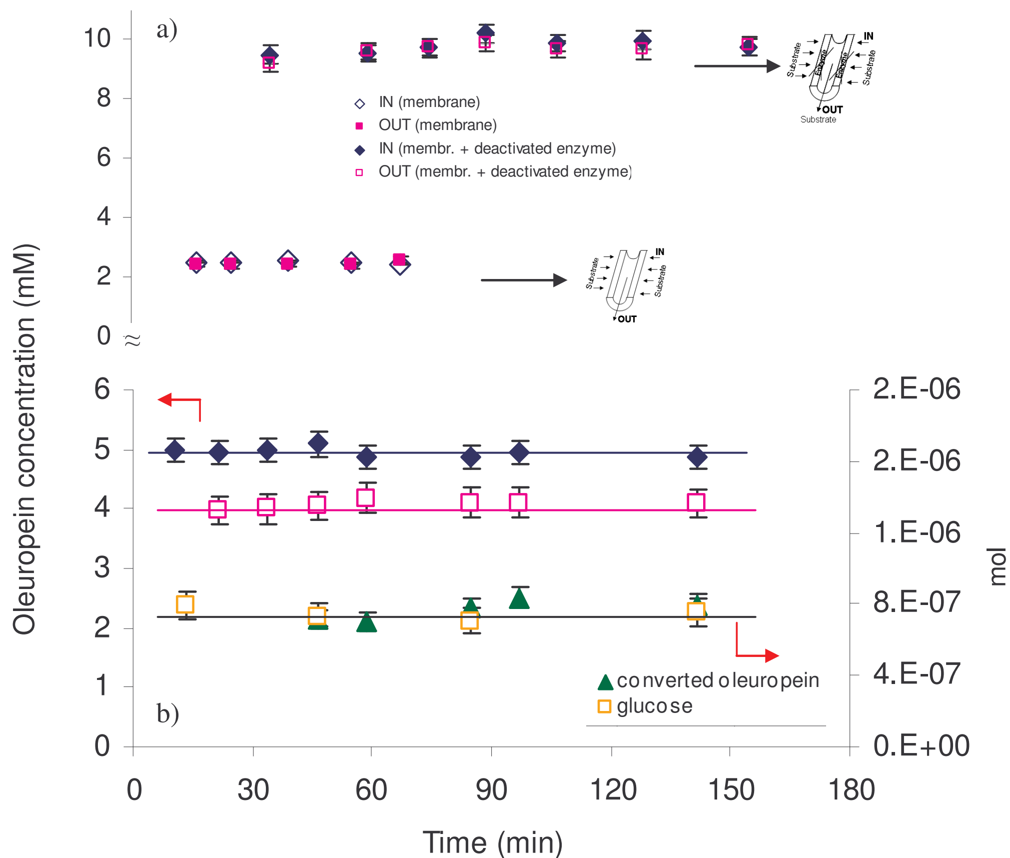


Fig. 7.5. Oleuropein concentration behaviour as a function of time. 5a) blank experiments where no enzyme or deactivated enzyme was present in the membrane. 5b) biocatalytic membrane reactor and relative correspondence to the reaction stoichiometry between mol of oleuropein converted (□) and mol of glucose produced (▲)

7.3.3.1 Enzyme-loaded membrane

Immobilization was carried out by using 3.5 mg of pure β-glucosidase from almond dissolved in 100 mL of 50 mM buffer at pH 6.5. The initial solution was circulated in the shell side of the membrane module at an axial velocity of 0.35 m/s and applying a transmembrane pressure of 0.3 bar. The enzyme solution permeated from shell to lumen side. To avoid enzyme denaturation, the feed, the module and the permeate solution were maintained at 4(±2) °C. The permeate flux during enzyme immobilization decreased as function of time as reported in Fig. 7.6. The absence of the protein in the permeate was confirmed by SDS-page electrophoresis (Fig. 7.6a). When the value of flux reached the steady-state, the immobilization process was stopped. Afterwards, the system was rinsed with buffer until no protein was detected (by measuring optical density at 280 nm) in the rinsing solutions.

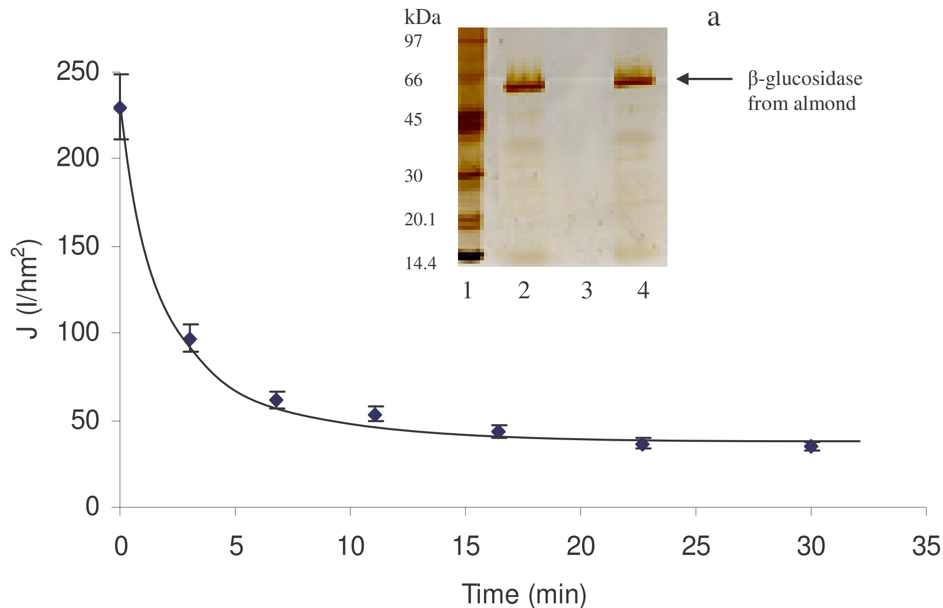


Fig. 7.6. Behaviour of flux as function of time during immobilization. a) SDS-page lane 1: marquer, lane 2: β-glucosidase from almond, lane: 3 permeate, lane 4: retentate

Due to the initial low protein concentration, an accurate measure of immobilized enzyme was difficult, basically because of difficulties in reproducibly detecting changes of protein concentration between initial and final solution as well as accurate estimation of protein in the rinsing solutions since it was below the test resolution limit. For this reason, a measure of hydraulic resistance was performed to confirm the presence of the protein within the membrane. As shown in Table 7.3, the protein present in the membrane caused an irreversible resistance of $1.34 \cdot 10^{12}$ (± $1.9 \cdot 10^{11}$) m^{-1}, calculated considering equations 3 and 4. It must be

pointed out that R_{irr} is overestimated due to low temperature used during permeate flux measurement with enzyme solution. Therefore, both amount of immobilised enzyme and promoted hydraulic resistance are rough estimations.

Table 7.3. Hydraulic resistance of membrane and fouling components for 30 kDa polysulphone capillary membrane

Membrane	Solution	Permeability (m/s·Pa)	Resistance	
			R	(m^{-1})
New membrane	Ultrapure water	$6.39 \cdot 10^{-10}$	R_m	$1.75 \cdot 10^{12}$ ($\pm 7.6 \cdot 10^{14}$)
Membrane during* immobilization	ß-glucosidase solution	$2.97 \cdot 10^{-10}$*	R_{TOT}	$3.78 \cdot 10^{12}$ ($\pm 2.5 \cdot 10^{15}$)
Membrane after immobilization and rinsing step	Ultrapure water	$3.64 \cdot 10^{-10}$	R_{irr}	$1.34 \cdot 10^{12}$ ($\pm 1.9 \cdot 10^{11}$)
-	-	-	R_f	$2.03 \cdot 10^{12}$ ($\pm 2.0 \cdot 10^{11}$)
-	-	-	R_{rev}	$6.9 \cdot 10^{11}$ ($\pm 6.9 \cdot 10^{10}$)

R_m= hydraulic resistance due to the membrane; R_{TOT}= hydraulic resistance due to membrane and fouling, R_f = hydraulic resistance due the overall fouling; R_{irr} = hydraulic resistance due to the irreversible fouling; R_{rev} = hydraulic resistance due to reversible fouling. *This value was calculated from the constant flux value as a function of time (see Fig 6)

Anyway, the hydraulic resistance due to irreversible fouling promoted by immobilized enzyme resulted roughly as high as the hydraulic resistance due to the polymeric membrane itself. Although this does not give the mass of immobilized enzyme, it indicates that the enzyme remained stably entrapped within the membrane.

The entrapment immobilization method provides a random distribution and orientation of the protein within the membrane, as it was confirmed by *in situ* detection of the enzyme using an adapted immunoassay method reported in our previous work [37].

Reaction rates were measured with both new prepared and re-used enzyme-loaded membranes. In the latter case, after each experiment, the efficiency of the enzyme-loaded membrane was verified by carrying out a reaction in the initial operating conditions.

The catalytic activity of the immobilized enzyme as a function of time was also evaluated, as described in Materials and Methods section. As shown in Fig. 7.7 the catalytic activity did

not show any significant decay for about 30 hours operation time. Test carried out after two months preservation at -80 °C confirmed that the enzyme was still active.

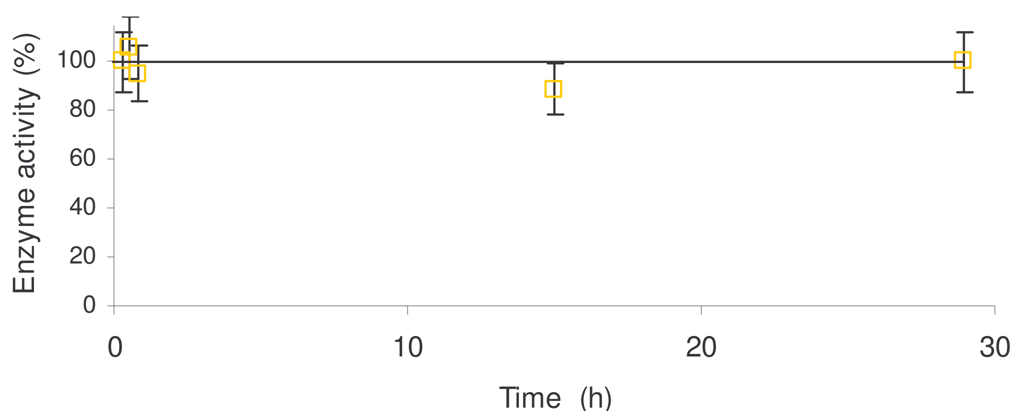


Fig. 7.7. Enzyme activity of immobilized β-glucosidase as a function of time. T = 25 °C, pH = 6.5, oleuropein concentration = 2 mM

7.3.3.2 Reaction kinetics with immobilized β-glucosidase

The enzymatic hydrolysis in the enzyme-loaded membrane reactor was carried out using 100 ml of oleuropein solution at pH 6.5 and 25°C. An example of time course of substrate concentration in the feed (IN) and permeate (OUT) solution through the biocatalytic membrane for an oleuropein initial concentration of 5 mM is reported in Fig. 7.5b. The figure also shows the stoichiometric correspondence between moles of oleuropein converted and glucose (and equivalent moles of aglycon) produced. The conversion was about 20 (±3) % for a residence time of about 126 s. During the experiment the reaction rate was about $2.5 \cdot 10^{-02}$ ($\pm 1 \cdot 10^{-3}$) $mmol/cm^3 \cdot h$ (calculated with equation 11).

The different conversion degree in STR (30%) and MBR (20%) was due to the lower residence time of the substrate in the biocatalytic membrane reactor, 126 s, compared with 9000 for STR.

Experiments at different substrate concentration (1, 2, 2.5, 5, 7.5, 10, 15 mM) were carried out to measure K_m and V_{max} for immobilized enzyme. Lineweaver-Burk plot is reported in Fig 7.4, and values of kinetic parameters are reported in Table 7.2. Obtained results are comparable to the one of the free native enzyme. The common observation in the literature

was that immobilized enzymes increase their stability while decreasing their catalytic activity, which is one of the major drawback that limits the application of the technology on a large scale. This study shows that the inverse relationship between stability and activity of immobilised enzymes is not a general rule. It is worth nothing that the demonstration has been made on the basis of kinetic parameters.

Results confirmed that the immobilization procedure did not alter the catalytic properties of the enzyme and that the system is suitable for further optimization and development at larger scale. For what concerns k_{+2} a thorough evaluation of its value was not possible, due to the difficulties in measuring the precise immobilized enzyme amount. On the other hand, on the basis of similar values of K_m and V_{max} between free and immobilized enzyme we can state that the immobilized enzyme is working with same performance, or even slightly better (low K_m and higher V_{max}) compared to the free enzyme. Then k_{+2} for the immobilized enzyme should be also of the same order as the one of the free enzyme. Assuming k_{+2} equal to the one for free enzyme the working immobilized enzyme can be calculated. In order to have a k_{+2} similar to the free enzyme, the working immobilized enzyme concentration should be around $7.05 \cdot 10^{-7}$ mmol/cm^3.

Table 7.2 also reports the k_{+2}/K_m ratio. This ratio expresses the enzyme efficiency. Either a large value of k_{+2} (rapid turnover) or a small value of K_m (high affinity for substrate) makes k_{+2}/K_m large. If we consider the k_{+2} value similar to the one of the free enzyme, the ratio between k_{+2}/K_m becomes larger for the biocatalytic membrane reactor, suggesting that the enzyme may have higher efficiency when immobilized.

7.4. Conclusions

In this work, the catalytic performance of a biocatalytic membrane reactor using β-glucosidase immobilized in 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. Kinetic parameters of β-glucosidase immobilized in the membrane reactor have been evaluated and compared with those of the ones of the free enzyme. Results evidenced that intrinsic kinetics of enzyme immobilized in the polysulfone membrane reactor was not negatively affected ($K_m = 3.80 \pm 0.14$ mM; $V_{max} = 0.046 \pm 0.002$ M·h^{-1}) compared to the free enzyme [$K_m = 4.80\ (\pm 0.11)$ mM, $V_{max} = 0.035\ (\pm 0.004)$ M·h^{-1};

$k_{+2} = 18$ ($\pm$ 0.9) s^{-1}]. On the contrary it seemed slightly improved, i.e lower K_m value indicates higher enzyme-substrate affinity. This shows that lower catalytic activity of immobilized enzyme, commonly observed in the literature, is not an intrinsic technological drawback and that appropriate control of biohybrid microstructured systems, microenvironment conditions and transport properties can even improve their catalytic efficiency.

References

[1] R. Briante, F. La Cara, F. Febbraio, R. Baroni, G. Piccialli, R. Carolla, P. Mainolfi, L. De Napoli, M. Patumi, G. Fontanazza, R. Nucci, Hydrolysis of oleuropein by rercombinant β-Glycosidase from Hyperthermophilic archaeon *Sulfolobus solfataricus* immobilized on chitosan matrix, J. Biotechnol. 77 (2000) 275-286.

[2] M. Esti, L. Cinquanta, E. La Notte, Phenolic Compounds in Different Olive Varieties. J. Agric. Food Chem. 46 (1998) 32-35.

[3] F. Paiva-Martins, M. H. Gordon, Isolation and characterization of the antioxidant Component 3, 4-Dihydroxyphenylethyl 4-formyl-3-formyl-3-formylmethyl-4-hexenoate from Olive (Olea europaea) leaves, J. Agric. Food Chem. 49 (2001) 4214-4219.

[4] I. Kubo, A. Matsumoto, I. Takase, A multichemica defense mechanism of bitter olive *Olea europaea* (oleaceae) J. Chem. Ecol. 11 (1985) 251-263.

[5] M. J. Amiot, A. Fleuriet, J. J.Macheix, Accumulation of oleuropein derivatives during olive maturation. Phytochemistry 28 (1989) 67-69.

[6] F. Visioli, G. Bellomo, G. Montedoro, C. Galli, Antioxidant and Other Biological Activities of Olive Mill Waste Waters, Atherosclerosis 117 (1995) 25-32.

[7] M. H. Gordon, F. Paiva-Martins, F. Almeida, Antioxidant activity of hydroxytyrosol acetate compared with that of other olive oil polyphenols, J. Agric. Food Chem. 49 (2001) 2480-2485.

[8] P. S. Rodis, V. T. Karathanos, A. Mantzavinou, Partitioning of Olive Oil Antioxidants between Oil and Water Phases, J. Agric. Food Chem. 50 (2002) 596-601.

[9] F. Paiva-Martins, M. H. Gordon, P. Gameiro, Activity and location of olive oil phenolic antioxidants in liposomes, Chem. Phys. Lipids 124 (2003) 23-36.

[10] M. N. Vissers, P. L. Zock, M. B. Katan, Bioavailability and antioxidant. effects of olive oil phenols in humans: a review, Eur. J. Clin. Nutr. 58 (2004) 955-965.

[11] M. Scigelova, S. Singh, D. H. G. Crout, Glycosidases- a great synthetic tool. J. Mol. Catal. B Enzym. 6 (1999) 483-494.

[12] H. Yeoh, Kinetic properties of Cassava ß-glucosidase immobilized in photo-crosslinkable resins. Biotechnol. Lett. 13 (1991) 697-700.

[13] A. Esen, ß-glucosidases: Biochemistry and Molecular Biology, American Chemical Society, Washington, 1993.

[14] M. A. Montero, A. Romeu, Properties of almond ß-glucosidase immobilized on concanavalin A-sepharose, Biochem. Mol. Biol. Int. 30 (1993) 685-689.

[15] Y. Hsuanyu, K. J. Laidler, Flow kinetics of immobilized ß-glucosidase. Biochem. Cell Biol. 64 (1986) 139-145.

[16] S. A. Backer, S. H. Doss, C. J. Gray, J. F. Kennedy, M. Stacy, T. H. Teo, β-glucosidase chemically bound to microcrystalline cellulose, Carbohydr. Res. 20 (1971) 1-7.

[17] V. R. Srinivasan, M. W. Bumm, Isolation and immobilization of ß-D-glucosidase from alcaligenes faecalis, Biotechnol. Bioeng. 16 (1974) 1413-1418.

[18] J. L. Iborra, M. R. Castellar, M Cànovas, A. Manjòn, Picrocicrin hydrolysis by immobilized β-glucosidase, Biotech. Lett. 14 (1992) 475-480.

[19] H. Nagamoto, Y. Matsushita, K. Sugamoto, T. Matsui, Preparartion and Properties of Gelatin-Immobilized ß-glucosidase from *Pirococcus furious*, Biosci. Biotechnol. 69 (2005) 128-136.

[20] R. Limiroli, R. Consonni, G. Ottolina, V. Marsilio, G. Bianchi, L. Zetta, J. Chem. Soc. Perkin Trans. 5(1995) 1519-1523.

[21] V. Marsilio, B. Lanza, N. Pozzi, Progress in table olive debittering: Degradation *in vitro* of
oleuropein and its derivatives by *Lactobacillus plantarum*, J Am Oil Chem Soc 73(1996) 593-597.

[22] R. Capasso, A. Evidente, C. Visca, L. Gianfreda, M. Maremoti, G. Greco, Production of Glucose and Bioactive Aglycone by Chemical and Enzymatic Hydrolysis of Purified Oleuropein from *Olea Europaea*, Appl. Biochem. Biotechnol. 61 (1997) 365-377.

[23] R. Briante, F. La Cara, M. P. Tonziello, F. Febbraio, R. Nucci, Antioxidant Activity of the Main Bioactive Derivates from Oleuropein Hydrolysis by Hyperthermophilic β-Glycosidase, J. Agric. Food Chem. 49 (2001) 3198-3203.

[24] R. Briante, M. Patumi, F. Febbraio, R. Nucci, Production of highly purified hydroxytyrosol from *Olea europaea* leaf extract biotransformed by hyperthermophilic β-glycosidase, J. Biotechnol. 111 (2004) 67-77.

[25] M. Guiso, C. Marra, Highlights in oleuropein aglycone structure. Nat. Prod. Res. 19 (2005) 105-109.

[26] E. Drioli, L. Giorno, Biocatalytic membrane reactors: Applications in the biotechnology and pharmaceutical industry. London: Taylor & Francis, 1999. p 51.

[27] L. Giorno, E. Drioli, Biocatalytic membrane reactors: applications and perspectives. Trends in Biotechnol. 18 (2000) 339-349.

[28] L. Giorno, R. Mazzei, E. Drioli, Biochemical membrane reactors in industrial processes, in Membranes Operations. Innovative separations and transformations, Eds. E. Drioli and L. Giorno, Wiley-VCH, in Press 2009. (ISBN-10 3-527-32038-5, www.wiley-vch.de)

[29] G. M. Rios, M. Belleville, D. Paolucci-Jeanjean, Membrane engineering in biotechnology: *quo vamus*? Trends Biotechnol. 25 (2007) 242-246.

[30] V.M. Balcão, A.L. Paiva, F.X Malcata, Bioreactors with immobilized lipase: State of the art. Enzyme and Microbial Technology, 18 (1996) 392-416.

[31] R. Valadez-Blanco, F. Castelo Ferreira, R. Ferreira Jorge, A. G. Livingston, Membrane bioreactor for biotransformations of hydrophobic molecules using organic solvent nanofiltration (OSN) membranes, J. Membr. Sci. 317 (2008) 50-64

[32] H.A. Sousa, C. Rodrigues, E. Klein, C.A.M. Afonso, J.G. Crespo, Immobilisation of pig liver esterase in hollow fibre membranes, Enzyme Microb. Technol. 29 (2001) 625.

[33] Z.M. Liu, J. Dubremez, V. Richard, Q. Yang, Z.K. Xu, P. Seta, Useful method for the spatial localization determination of enzyme (peroxidase) distribution on microfiltration membrane, J. Membr. Sci. 267 (2005) 2.

[34] A. Ranalli, S. Contento, L. Lucera, M. Di Febo, D. Marchigiani, V. Di Fonzo, Factors Affecting the Contents of Irinoids Oleuropein in Olive Leaves (Olea europaea L.) J. Agric. Food Chem. 54 (2006) 434-440.

[35] P. Aimar, J. Howell, Effect of concentration polarization Boundary Layer Development on the Flux Limitations in Ultrafiltration, Chem. Eng. Res. Des. 67 (1989) 225-261.

[36] J. E. Bailey, D. F. Ollis, Biochemical engineering fundamentals. New York: McGraw-Hill. 1986, p 97

[37] S. Mazzuca, L. Giorno, A. Spadafora, R. Mazzei, E. Drioli, Immunolocalization of ß-glucosidase immobilized within polysulphone capillary membrane and evaluation of its activity *in situ*, J. Memb. Sci. 285 (2006)152–158.

Chapter 8

Biocatalytic membrane reactor and membrane emulsification concepts combined in a single unit to assist production and separation of water unstable reaction products

Abstract

The aim of the present work was to design and proof the concept of an integrated and intensified membrane system able to carry out in a single operation unit, bioconversion in water phase, and simultaneous separation of reaction products having different solubility and stability in water, by creating water in oil emulsion. To achieve this aim, the concepts of biocatalytic membrane reactor and membrane emulsification have been integrated so that to build-up a combined membrane operation system where hydrolysis occurred within the microporous membrane structure (that contained immobilized enzyme) and extraction occurred at the membrane interface where the permeating water reaction phase was collected as droplets into the organic phase circulated along the lumen membrane surface. The oleuropein hydrolysis into glucose and isomer of oleuropein aglycon (3,4-DHPEA-EA) by means of β-glucosidase immobilized in porous polymeric membrane has been used as a model reaction system.

8.1. Introduction

There is a large number of organic compounds of interest for the chemical and biotechnological industries which have low solubility in water. Biotransformations can offer an attractive alternative to conventional chemistry for the production of such specialty chemicals. Most industrial processes using biocatalysts involve compounds which are readily soluble in water. However many commercially viable biotransformations involve poor water soluble compounds, which can lead to poor volumetric productivities in single phase aqueous bioreactors.

A special and important field that gained interest in biotransformation is the enzyme/substrate system β-glucosidase/oleuropein, because the main important phenolic compounds contained in olive oil, are secoiridoids derivates coming from oleuropein hydrolysis. Different compounds are produced during the oleuropein hydrolysis, and they are partitioned into oil or water phase, basing on their partition coefficient.

The prevalent phenolic compounds in olive oil are secoiridoid derivates of oleuropein such as an isomer of oleuropein aglycon (3,4-DHPEA-EA, 3,4-DiHydroxyPhenyl Ethanol – Elenolic acid) the dialdehydic form of elenolic acid linked to 3,4-(dihydroxyphenyl) ethanol (3,4-DHPEA-EDA, 3,4-DiHydroxyPhenyl Ethanol - Elenolic acid Di-Aledehyde) (Fig. 8.1) and *p*-hydroxyphenylethanol, these molecules are of particular significance because of their strong antioxidant activity in several lipid systems including oil [1] [2].

One of the current problems for developing rapid and reproducible analysis of phenolic compounds is the absence of suitable pure standards, in particular secoiridoids molecules [3].

To understand the complete molecular transformation of oleuropein taking place in the olive during fruit ripening or deterioration during crushing and malaxation by the intervention of natural β-glucosidase the biphasic liquid system that naturally occur in olives must be considered. Bianco et al. [4] reported the NMR characterization of the complete sequence of oleuropein metabolites during agrifood processing carrying out the β-glucosidase reaction in biomimetic conditions. In the mentioned work it is described that when the reaction was carried out in $D_2O/CDCl_3$, the compounds present in the organic fraction were: ***1*** (3,4-DHPEA-EA) and ***2*** (3,4-DHPEA-EDA) (Fig. 8.1), showing that these molecules are derivates from oleuropein hydrolysis. In addition the work demonstrated that lipid/water interface promotes the rapid aldehydes rearrangement into elenolates within 5 min. The biomimetic experiment allowed the detailed description of the complete molecular transformation sequence occurring during the oleuropein enzymatic hydrolysis [4]. The two important compounds (3,4-DHPEA-EA and 3,4-DHPEA-EDA) are not found in the reaction product coming from β-glucosidase hydrolysis conducted in water phase due to their low water solubility.

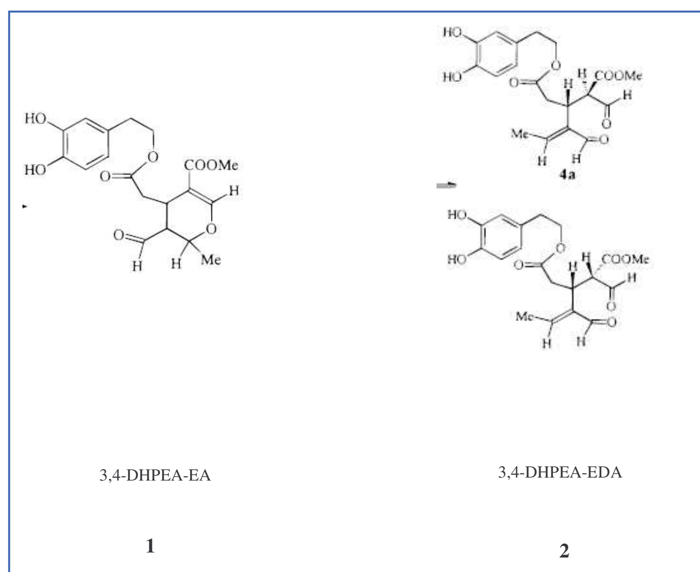


Fig. 8.1. Structures of 3,4-DHPEA-EA (3,4-DiHydroxyPhenyl Ethanol – Elenolic acid) and 3,4-DHPEA-EDA (3,4-DiHydroxyPhenyl Ethanol - Elenolic acid Di-Aledehyde).

Another work [5] demonstrated that, when the oleuropein hydrolysis was carried out in water phase, the oleuropein aglycon produced in the first step of the reaction, it is fastly rearranged into water stable products because of its low water solubility.

Rodis et al. [6] also determined the partition coefficient (K_p= C_{oil}/C_{water}) of the natural phenolic compounds between aqueous and olive oil phase, demonstrating that both 3,4-DHPEA-EDA (K_p= 0.189) and in particular 3,4-DHPEA-EA (K_p= 1.49) have higher partition coefficient compared to oleuropein (K_p= 0.0006).

Basing on the considerations previously mentioned about the solubility of 3,4-DHPEA-EA and on its antioxidant important properties in olive oil, the aim of the present work was the development of membrane bioreactor configuration able to simultaneously produce the water unstable compound 3,4-DHPEA-EA and promote its partition into an organic phase that does not permit its transformation into the water stable compounds.

For the production and the simultaneous extraction of 3,4-DHPEA-EA, ***1,*** it should be needed a system in which it is possible to have the hydrolysis reaction in water phase and then its simultaneous contact with an organic phase: the water phase contains the hydrosoluble compounds (glucose and unreacted oleuropein) while the organic phase extracts and isolates the compounds not completely soluble in water and in particular 3,4-DHPEA-EA.

The different compartments present in the biocatalytic membrane reactor working under permeating conditions, permitted to conduct the enzymatic hydrolysis in water and then disperse the water permeating through the biocatalytic membrane (containing hydrolysis products) into an organic phase able to extract the isomer of oleuropein aglycon.

The reaction occurred within the porous membrane where the enzyme was immobilized by entrapment. The oleuropein aqueous solution was fed to the enzyme-loaded membrane by

convective flow from shell to lumen. The organic phase was circulated along the lumen. The water/organic interface was formed at the lumen side basing on membrane emulsification concept.

The possibility to conduct the oleuropein hydrolysis with commercial β-glucosidase heterogenized on polysulphone membrane was already demonstrated in a previous work [7], where the biocatalytic membrane performance was evaluated in terms of kinetic properties.

In this work the system was integrated to intensify the process by coupling the productive biocatalytic membrane reactor with an extractive contactor process promoted by membrane emulsification concept.

8.2. Materials and methods

8.2.1. Chemicals

The β-glucosidase from almond (chromatographically purified lyophilized powder, Cod G4511, one unit will liberate 1.0 μmole per min of glucose from salicin at pH 5.0 at 37 °C) and glucose were obtained from Sigma Aldrich, oleuropein from Extrasynthese (France). Acetic acid (Carlo Erba) and sodium acetate (Sigma Aldrich) were used to prepare 50 mM acetate buffer pH 6.5. Acetonitrile and *o*-phosphoric acid for HPLC mobile phase preparation were purchased from Carlo Erba and Sigma Aldrich, respectively. $CHCl_3$, CH_3OH and silica gel 60 F254 plates were purchased from Merck. Limonene was purchased from Sigma-Aldrich.

8.2.2. Methods

Oleuropein was measured by HPLC with an adapted method from the one described by [8]. A reverse silica Adsorbosphere XL C18 column, 250-4.6 mm, 5 μm (Grace) was used. The mobile phase was a mixture of acetonotrile/water (21:79) acidified with *o*-phosphoric acid (up to pH 3). The flow rate and pressure were 1.2 ml/min and 145 (± 4) bar, respectively. The volume of sample injection was 20 μl. The oleuropein was detected at 280 nm wavelength.

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. Oleuropein and its aglycon form in water were qualitatively evaluated by TLC as the method described in [9]. In that work it is shown that the compounds detectable by TLC after oleuropein hydrolysis are: unreacted oleuropein, oleuropein aglycones and in some case also hydroxytyrosol. The absence of hydroxytyrosol in our samples coming from the reaction was verified by HPLC,

according to the following method: reverse silica LiChoCART Superspher RP8, (250-4 mm, 5 µm, Merk) and acetonitrile/ water (10/90) as mobile phase. The flux and the pressure were 1 ml/min and 212 bar, respectively. The samples were injected with a 50% of methanol.
TLC was carried out on silica gel 60 F_{254} plates and was used to qualitatively monitor the oleuropein aglycon formation. The spots on silica plates were revealed under UV light.
Since oleuropein aglycons forms in water are not commercially available, an estimation of its concentration in reaction samples was calculated by HPLC using oleuropein as external standard. The pick of oleuropein aglycons were identified conducting the reaction in batch system at 25 °C, pH 6.5 for 54 hours.

8.2.3 Equipments

To produce and simultaneously extract the isomer of oleuropein aglycon (3,4-DHPEA-EA), the oleuropein hydrolysis by β-glucosidase action, was conducted in a system where both the water and oil could simultaneously meet after the reaction occurred. The presence of water assured the oleuropein hydrolysis and the recovery of glucose, while the presence of oil assured the extraction of the isomer of oleuropein aglycon.
Limonene was used as the organic phase due to its known properties in food applications as food preservative [10].
The new system developed to produce and simultaneously extract the compound of interest from the product mixture is composed by an enzyme-loaded membrane reactor coupled with an extractive contactor based on membrane emulsification concept. The intensified system is illustrated in Fig 8.2. The oleuropein aqueous phase is forced to pass through the enzyme-loaded membrane. Here the reaction occurs, then the products and the unreacted substrate present in water phase coming from the biocatalytic membrane meet the organic phase on the lumen side. Since the two phases are immiscible, water-in-oil droplets are formed. The droplets size is regulated so that emulsion stability is not too high. In this way, the two phases easily separate obtaining readily suitable phases with purified products.
A monophasic stirred tank reactor (STR) [7] was used to carry out the reaction in batch water phase, useful to visualize the product obtained in water and as reference system.
To better quantify the aglycon produced, in the present work the reaction of oleuropein hydrolysis in batch was conducted for longer time (up to about 60 h).

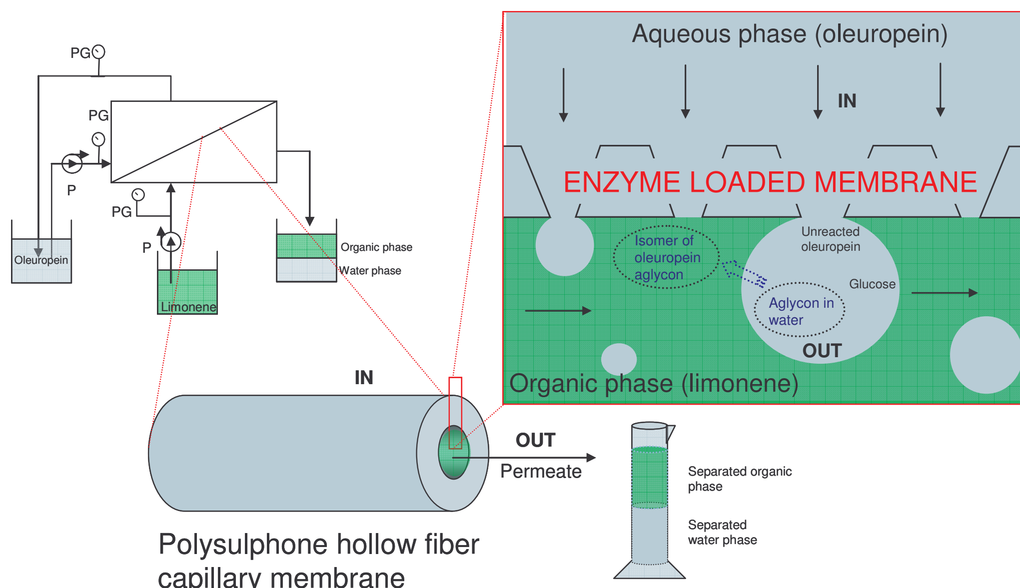


Fig. 8.2. Experimental apparatus used to carry out oleuropein hydrolysis in the combined biocatalytic membrane reactor and membrane emulsification system (BMR-ME) (limonene/aqueous phase). P: peristaltic pump, PG: pressure gauges

8.2.3.1. Biocatalytic membrane reactor and coupled extraction via membrane emulsification (BMR-ME)

The equipment used to carry out the hydrolysis reaction in water and the extraction by means of membrane emulsification in a single step is illustrated in Fig. 8.2. It consists of a membrane module made of a pyrex cylinder as shell containing four polysulphone capillary membranes of 30 kDa nominal molecular weight cut-off (NMWCO) (PS 30 kDa) and internal/external diameter of 1.08/1.75 mm. The membranes have asymmetric structure with the selective layer on the lumen side and the sponge layer on the shell side. The membrane module effective length was 10.5 (± 0.5) cm and the internal/external membrane surface area was $1.46 \cdot 10^{-3}/2.36 \cdot 10^{-3}$ ($\pm 4 \cdot 10^{-4}$) m^2, and a membrane void volume was 0.54 (± 0.02) cm^3. This represents the membrane reactor volume. The system is constituted also by a tank containing the oleuropein solution, a peristaltic pump to feed the reagent solution to the biocatalytic membrane reactor, pressure gauges to measure inlet and outlet pressure and a graduated cylinder to collect permeate solution containing reaction product.

When the reaction is conducted in the coupled membrane bioreactor/membrane emulsification system a stream of organic phase was fed along the lumen side. The flow rate of the organic phase was 0.24 ml/min. The water phase permeating through the biocatalytic membrane met the organic phase in the lumen side forming the droplets. They were then collected and separated spontaneously after short time. After the separation the water phase present in the samples was then analyzed by TLC and HPLC as previously described.

8.2.3.2. Reaction operation mode in the combined biocatalytic membrane reactor and membrane emulsification system

After the enzyme was immobilized within the membrane by entrapment (Mazzei et al 2009) 100 ml of oleuropein solution, in buffer at pH 6.5, was fed to the shell circuit and permeated through the membrane thickness (where the enzyme was loaded) with an applied transmembrane pressure of 0.1 bar and a permeate flow rate of 0.30 (±0.05) ml/min. A thermostatic bath was used to keep the temperature constant at 25°C. As oleuropein (540.23 Da) passed through the enzyme-loaded membrane it was converted into the corresponding reaction products, aglycons and glucose.

To directly compare the production/extraction of isomer of oleuropein aglycon, the oleuropein hydrolysis was initially carried out for 80 min in the biocatalytic membrane reactor system (without circulating the organic phase in the lumen side) and then for subsequent 80 min in the coupled BMR-ME system. Each reported data is the average value of at least three experiments.

8.3. Results

8.3.1. Stirred tank reactor

The oleuropein hydrolysis carried out in a stirred tank reactor using free β-glucosidase was used as a control reaction system. Furthermore, the reaction was carried out for long time (about 60 hours) in order to achieve higher aglycon production to be used to better identify and quantify it. The reaction was carried out with 2.5 mM initial oleuropein concentration.

In Fig. 8.3 the substrate decrease and product formation are reported. The upper part of the figure confirms that results were congruent with the reaction stoichiometry, from which it is known that one mol of oleuropein gives one mol of aglycons and one of glucose.

An overall conversion degree of 70 (± 3) % was reached after 53 h of residence time and the initial reaction rate was about $2 \cdot 10^{-4}$ mmol/cm^3 h.

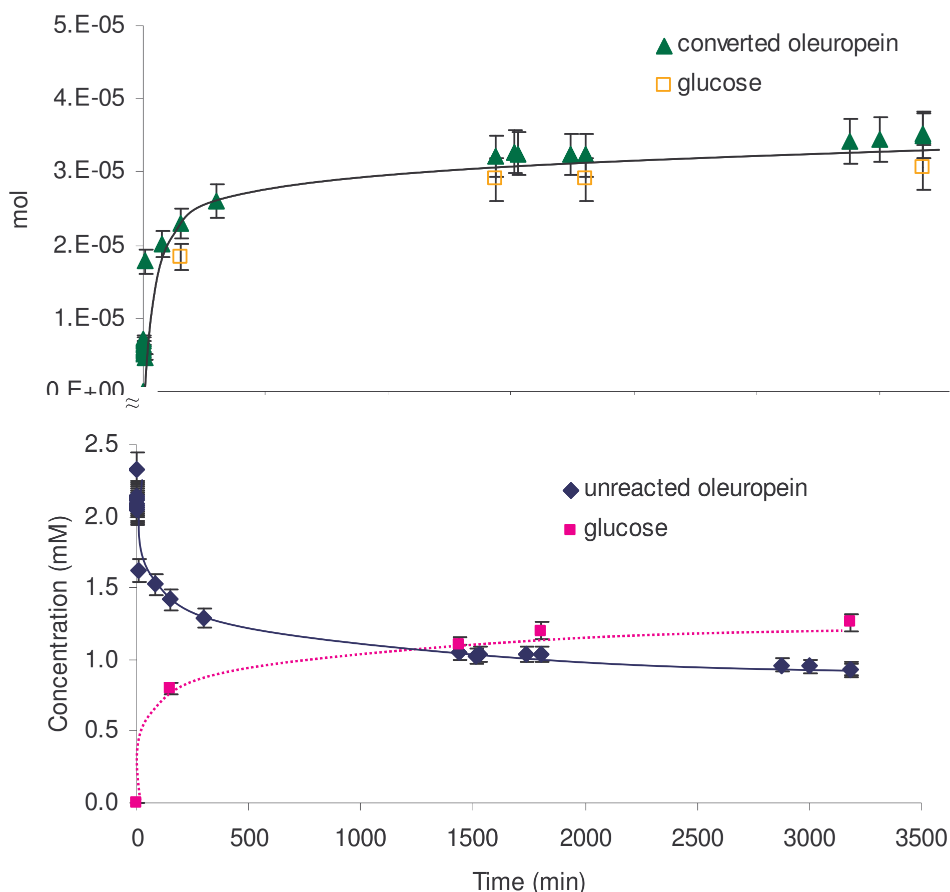


Fig. 8.3. Behaviour of unreacted oleuropein (◆) and glucose concentration (■) as function of time in the stirred tank reactor and relative correspondence to the reaction stoichiometry between mol of converted oleuropein (▲) and mol of glucose produced (□).

Fig. 8.4a shows the HPLC chromatogram obtained from enzymatic reaction samples analyses. In addition to the oleuropein peak, the figure shows another peak which resulted to correspond to the oleuropein aglycon. The oleuropein analysed with the condition explained before has a retention time of 12.11 min. The peak with retention time 2.9 corresponds to the oleuropein aglycon. In fact, it increased its concentration as a function of time in reaction samples which indicated that it is a reaction product. It is not hydroxytyrosol (as it was verified by the previously described method that no hydroxytyrosol was formed) and it is not glucose nor β-glucosidase as it was verified by injecting their standard solution in control analyses.

Fig. 8.4b also reports the results obtained by TLC analyses. The spot corresponding to the oleuropein was identified by using its own standard. The upper spot has been attributed to the aglycon according to TLC reported by Briante et al [9] and on the basis of the fact that no other component present in the reaction mixture (glucose and β-glucosidase) was detectable by TLC analyses. About hydroxytyrosol, as previously mentioned, it was not produced in this reaction. The figure shows that there is a decrease of oleuropein amount and a corresponding

increase of aglycon amount as a function of time. Results and literature information permitted to deduce that the compound evidenced on TLC that increased its concentration as a function of time was oleuropein aglycon.

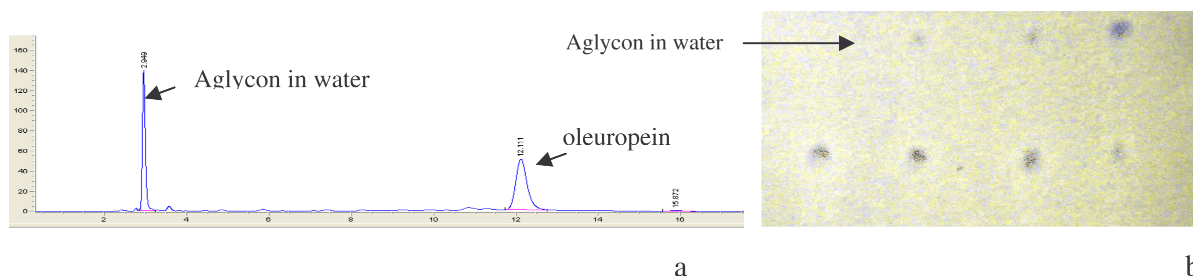


Fig. 8.4. Qualitative evaluation of product formation during oleuropein hydrolysis in stirred tank reactor as a function of time. a) HPLC chromatogram of reaction sample. b) TLC (80/20 chloroform/methanol, pH 6.5, 25°C) Column 1: standard of oleuropein; columns 2, 3, 4 samples of oleuropein hydrolysis as a function of time (6 min, 80 min, 320 min, respectively).

Based on the evidence that the aglycon molecule was present in the samples and knowing that its moles were equivalent to that of glucose and reacted oleuropein, we used this information and data to build up calibration curve of oleuropein aglycon. This calibration curve was used to quantify aglycon in water phase during reaction/extraction in subsequent experiments carried out with the intensified BMR-ME system.

8.3.2. Biocatalytic membrane reactor and membrane emulsification coupled system

The substrate concentration in the feed (IN) and permeate (OUT) solution through the biocatalytic membrane for an oleuropein initial concentration of 2.5 mM is reported in Fig. 8.5.

The system operated for 80 minutes as a biocatalytic membrane reactor operating in water and for 80 minutes as a biocatalytic membrane reactor operating in water combined with a water/oil cross-flow membrane emulsification process.

The constant concentration of the converted oleuropein indicates constant performance of the continuous (time invariant) reactor system for both operating conditions (absence or presence of organic phase in the lumen side allowing water in oil droplet formation for lypophilic aglycon extraction). The figure also shows the stoichiometric correspondence between moles of oleuropein converted and glucose produced as a function of time. Results confirmed that

oleuropein and glucose remained in the water phase and were not affected by the presence of the organic phase.

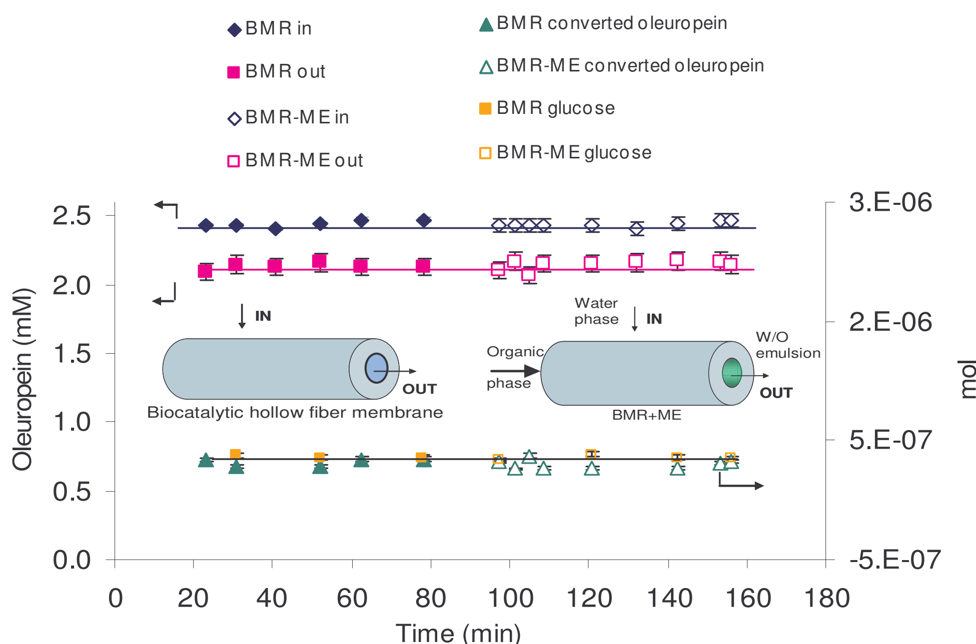


Fig. 8.5. Oleuropein concentration behaviour as a function of time in the biocatalytic membrane reactor (BMR) and in the coupled system (BMR-ME) and relative correspondence to the reaction stoichiometry between mol of oleuropein converted (▲, △) and mol of glucose (■, □) produced in the two systems, respectively

A conversion degree of 20% (± 5) was reached for a residence time of 126 s.

Fig. 8.6 shows the behaviour of oleuropein aglycon together with moles of converted oleuropein and glucose as a function of time, during the operation of biocatalytic membrane reactor in water and subsequent combination with membrane emulsification by circulation the organic phase along the lumen side with formation of water in oil droplets. The concentration of aglycon was measured by HPLC as previously mentioned.

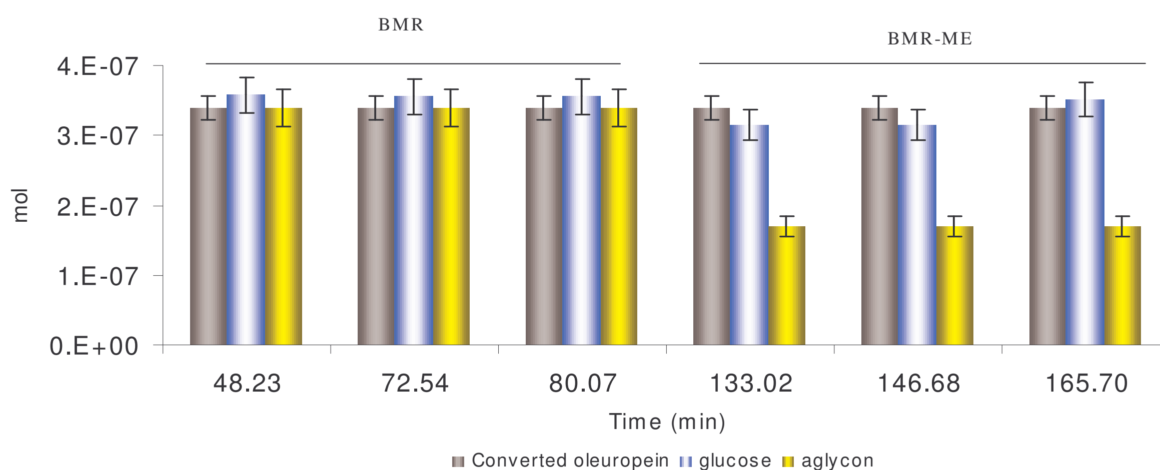


Fig. 8.6 Mol of converted oleuropein, glucose and aglycon in the biocatalytic membrane reactor (BMR) and in the coupled system (BMR-ME) as a function of time.

The figure shows that during the first 80 min the behaviour of aglycon amount is equivalent to that of glucose and converted oleuropein. Afterwards, when the circulation of organic solvent along the lumen was started, the amount of aglycon in water was about halved compared to glucose and converted oleuropein. This was obtained thanks to the extraction of isomer of oleuropein aglycon into the organic phase. The extraction capacity depended on the isomer of oleuropein aglycon partition coefficient between water and used organic phase as well as on the droplet size which controlled the water/oil interface area and the droplet stability, i.e. the contact time between the two phases before the emulsion separated. The extraction of the aglycon can be increased by properly adapting these conditions. The present work aimed at proving the concept of the proposed BMR-ME intensified membrane system with which it has been possible to produce in water phase and simultaneously extract, stabilize and purify the isomer of oleuropein aglycon in organic phase.

8.4. Conclusions

Results showed that using the combined biocatalytic membrane reactor and membrane emulsification system, hydrolysis was performed with the same efficiency as in control experiments carried out with the either free or immobilized enzyme in monophase water system. Furthermore the hydrophilic components (glucose and non reacted oleuropein) were kept in water phase whilst the lipophilic molecule (aglycon) was extracted into the organic phase.

The process evidenced the possibility to obtain the non commercially available isomer of oleuropein aglycon (3,4-DHPEA-EA) in pure form.

The proposed concept also opens for strategies able to control intermediate reaction products sequestration in multistep reaction systems.

References

[1] F. Paiva-Martins, M.H Gordon., Isolation and characterization of the antioxidant component 3,4-dihydroxyphenylethyl 4-formyl-3-formylmethyl-4-hexenoate from olive leaves, J. Agric. Food Chem. 49 (2001), 4214–4219

[2] G. Montedoro, M. Servili, M. Baldioli, R. Selvaggini, E. Miniati and A. Macchioni, Simple and hydrolyzable phenolic compounds in virgin olive oil. 3. Spectroscopic characterization of the secoiridoid derivatives. J. Agric. Food Chem. 41 (1993), 2228–2234

[3] A. Bendini, L. Cerretani, A. Carrasco-Pancorbo, A.M. Gòmez-Caravaca, A. Segura-Carretero, A. Fernàndez-Gutierrez, G. Lerker, Phenolic molecole in virgin olive oils: a survey of their sensory properties, healt effects, antioxidant activity and analytical methods. An overview of the last decade, Molecules 12 (2007) 1679-1719.

[4] A. D. Bianco, A. Piperno, G. Romeo, N. Uccella, NMR Experiments of Oleuropein Biomimetic Hydrolysis, J. Agric. Food Chem., 47 (1999), 3665-3668.

[5] M. Guiso, C. Marra, Highlights in oleuropein aglycone structure. Nat. Prod. Res. 19 (2005) 105-109

[6] P. S. Rodis, V. T. Karathanos, A. Mantzavinou, Partitioning of Olive Oil Antioxidants between Oil and Water Phases, J. Agric. Food Chem. 50 (2002) 596-601.

[7] R. Mazzei, L. Giorno, E. Piacentini, S. Mazzuca, E. Drioli, Kinetic study of a biocatalytic membrane reactor containing immobilized β-glucosidase for the hydrolysis of oleuropein J. Membr. Sci., 339 (2009) 215-223

[8] A. Ranalli, S. Contento, L. Lucera, M. Di Febo, D. Marchigiani, V. Di Fonzo, Factors affecting the contents of irinoids oleuropein in olive leaves (*Olea europaea* L.), J. Agric. Food Chem. 54 (2006) 434–440.

[9] R. Briante, F. La Cara, F. Febbraio, R. Baroni, G. Piccialli, R. Carolla, P. Mainolfi, L. De Napoli, M. Patumi, G. Fontanazza, R. Nucci, Hydrolysis of oleuropein by rercombinant β-Glycosidase from Hyperthermophilic archaeon *Sulfolobus solfataricus* immobilized on chitosan matrix, J. Biotechnol. 77 (2000) 275-286.

[10] M. Maròstica Junior, T. A. A. Rocha e Silva, G.C. Franchi, A. Nowill, M. Pastore, S. Hyslop, Antioxidant potential of aroma compounds obtained by limonene biotrasformation of orange essential oil, Food Chem. 116 (2009) 8-12.

Conclusions

The research work carried out aimed at developing biohybrid systems, based on the integration of vegetal plant biomolecules and artificial membranes, able to simulate *in vitro* efficient biological processes. The compartimentalization present *in vivo* was created by a bioartificial membrane system able to contain biological agents and produce high added value compound under controlled conditions. The studied compartimentalization mimiking biological system concerned two mechanism in which β-glucosidase was involved: defence mechanism against pathogens, and production of phenolic compounds. This last case was taken as example to develop the bioartificial system, being phenolic compounds important antioxidant molecules, some of them not commercially available yet, and in view of the potential application that the isolated substances can have in pharmaceutical and food field.

The advances promoted in the present work include:

1) Development of biocatalytic membranes with either heterogenized natural β-glucosidase from olive fruit or commercial β-glucosidase from almond.

2) Production of β-glucosidase from olive fruit not commercial available as purified extract by membrane processes. The overall results obtained showed that the β-glucosidase functional stability was preserved during the membrane operations and the removal of 20 kDa proteins allowed to increase the specific activity of about 52 % compared to the ones present in the initial fruit extract.

3) The biocatalytic membrane with heterogenized enzyme (from olive fruit or from almond) were evaluated by new combined method detection, able to localize the sites of enzyme immobilization on the membrane and to determine its catalytic activity *in situ*. The described approach can be useful to clarify crucial information for the optimization of enzyme immobilization techniques.

4) Evaluation of catalytic properties of the enzyme heterogenized in polymeric membrane system and measurement of kinetic parameters (K_m, V_{max}, k_{+2}). In particular the kinetic performance of β-glucosidase immobilized in the membrane reactor have been evaluated and compared with those of the one of the free enzyme. Results evidenced that the intrinsic kinetics of enzyme immobilized in the polysulphone membrane reactor was not negatively affected. This shows that lower catalytic activity of immobilized enzyme commonly observed in the literature, is not an intrinsic technological drawback and that appropriate control of biohybrid microstructured system, microenvironment conditions and transport properties can even improve their catalytic efficiency.

5) Development of an innovative bioartificial membrane system for the hydrolysis of oleuropein and the simultaneous production and isolation of antioxidant phenolic compounds not stable in water but of great importance for its potential application, if produced in pure form, in food and pharmaceutical field. The system simulated not only the different compartimentalization present in *in vivo* plant system, but also the different phases that are present in olives and during olive oil production. The bioartificial system combined two technologies, biocatalytic membrane reactors and membrane emulsification process. The integrated/intensified bioartificial system is able to carry out in a single unit bioconversion and extraction of water unstable compounds in organic phase.

The process evidenced the possibility to obtain the isomer of oleuropein aglycon in pure form, a very important molecule not commercially available and of high interest in pharmacological and food field, because of its high antioxidant properties known from *in vivo* studies.

Appendix

- Awards from 2006 to 2009

1. **“Travel award”** to participate to NanoMemCourse “Synthesis and characterization ”, Saragoza, Spain, 7-16 Novembre 2007

2. **“Travel award”** to participate to NanoMemCourse “Nanostructured materials, membrane modelling and simulation”, Patrasso, Grecia 18-27 Giugno 2008.

3. **“Best oral presentation”** to participate to “9th International conference on Catalysis in membrane Reactors” “Biocatalytic membrane Reactor to produce bioactive high value compounds from olive mill waste water: a kinetic analysis. **R.Mazzei**, L.Giorno, S.Mazzuca, E.Drioli, 9th ICCMR9 2009, Lione, Francia.

4. **“Travel award”** to partecipate to Euromembrane 2009, Monpellier, Francia, 6-10 Settembre 2009

5. **Co-author of awarded presentation**
   EMS Prize for the best oral presentation at Euromembrane 2006 “Distribution of phase transfer biocatalyst at the oil/water interface by membrane emulsifier and evaluation of enantiocatalytic performance”, L. Giorno, E. Piacentini, **R. Mazzei**, E. Drioli.

- Relevant publications from 2006 to 2009

1) **R. Mazzei**, L. Giorno, E. Piacentini, S. Mazzuca, E. Drioli, Kinetic study of a biocatalytic membrane reactor containing immobilized -glucosidase for the hydrolysis of oleuropein, Journal of Membrane Science, 339, (2009) 215-223.

2) **R. Mazzei**, L. Giorno, E. Piacentini, E. Drioli. Symbiosis, Advances in Biocatalytic Membrane Reactors for the production of non commercially available pharmacologically active compounds from vegetal material, New Biotechnology, (2009) 25S, p. S276.

3) L. Giorno, **R. Mazzei,** E. Drioli, Biochemical membrane reactors in industrial processes in Membranes Operations: Innovative separations and transformations, Eds. E. Drioli, L. Giorno, Wiley-VCH,Weinheim, 2009, p. 397.

4) E. Piacentini, L.Giorno, **R.Mazzei**, E.Drioli, New development for the controlled fabrication of microstructured multiphase bioreactor using membrane emulsification technology, New Biotechnology, (2009) 25S, pp S168.

5) **R. Mazzei**, L. Giorno, A. Spadafora, S. Mazzuca, E. Drioli, Improvement of beta-glucodsidase activity of Olea europaea fruit extracts processed by membrane technology, *Korean membrane Journal*, 8(1), 2007, 58-66.

6) **R. Mazzei**, L. Giorno, S. Mazzuca, A. Spadafora, E. Drioli, beta-glucosidase separation from Olea europaea fruit and its use in membrane bioreactors for hydrolysis of oleuropein, Desalination 200 (2006) 483-484.

7) S. Mazzuca, L. Giorno, A. Spadafora, **R. Mazzei**, E. Drioli, A new combined method to localize enzyme immobilized in polymeric membranes and evaluate its activity *in situ*, Desalination 199 (2006) 228-229

8) S. Mazzuca, L. Giorno, A. Spadafora, **R. Mazzei**, E. Drioli, Immunolocalization of β-glucosidase immobilized within polysulphone capillary membrane and evaluation of its activity in situ, *Journal of Membrane Science* 285 (2006) 152–158.

Other publications

9) L. Giorno, E. Piacentini, **R. Mazzei**, E. Drioli, Distribution of phase transfer biocatalyst at the oil/water interface by membrane emulsifier and evaluation of enantiocatalytic performance, Desalination 199 (2006) 182-184

10) L. Giorno**,** E. D'Amore, **R. Mazzei**, E. Piacentini, J. Zhang, E. Drioli, R. Cassano, N. Picci, An innovative approach to improve the performance of a two separate phase enzyme membrane reactor by immobilizing lipase in presence of emulsion, *Journal of Membrane Science*, 295 (2007) 95-101

11) Seung-Hak CHOI, Francesco SCURA, Giuseppe BARBIERI, **Rosalinda MAZZEI**, Lidietta GIORNO, Enrico DRIOLI, Jeong-Hoon KIM, Bio-degradation of Phenol in Wastewater by Enzyme-loaded Membrane Reactor: Numerical Approach, *Membrane Journal,* 19 (2009) 72-82.

12) L. Giorno, E. Piacentini, **R. Mazzei**, E. Drioli. Membrane emulsification as a novel method to distribute phase-transfer biocatalysts at the oil/water interface in bioorganic reactions. *Journal of Membrane Science* 317, 19-25, 2008

- Oral presentation to International congress 2006/2009

- Silvia Mazzuca, Lidietta Giorno, Antonia Spadafora, **Rosalinda Mazzei,** Enrico Drioli. A new combined method to localize enzyme immobilized in polymeric membranes and evaluate its activity in situ, presented at Euromembrane 2006, Taormina, Italia.

- **R. Mazzei**, L. Giorno, S. Mazzuca, E. Drioli. Biocatalytic membrane reactor to produce high value compounds from olive mill waste: a kinetic analysis, presented at International Conference of Membrane Reactors, ICCMR9 2009, Lyon, Francia.

-**R. Mazzei,** L. Giorno, E. Drioli**.** Biocatalytic membrane reactor to guide and control bioconversion, isolation and stabilization of water unstable intermediates**,** presented at Euromembrane 2009, Montpellier, Francia

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Proceedings

R. Mazzei, L.Giorno, E. Drioli, Biocatalytic membrane reactor to guide and control bioconversion, isolation, and stabilization of water unstable intermediates, Euromembrane 2009, Montpellier, France

R. Mazzei, L. Giorno, S. Mazzuca, E. Drioli, Biocatalytic membrane reactor to produce bioactive high value compounds from olive mill waste water: a kinetic analysis, International Conference of Catalysis in Membrane Reactors, ICCMR9, 2009, Lyon, France

L. Giorno, **R. Mazzei**, E. Piacentini, S. Chakraborty, E. Drioli, Membrane Bioreactors for production of nutraceuticals, *IMeTI* Workshop on "Membrane Applications in Agrofood" 2009, Cetraro (CS), Italy

Rosalinda Mazzei, Lidietta Giorno, Enrico Drioli, Integration of biocatalytic membrane reactor and membrane emulsification concepts for advanced systems in food by-products valorisation, *IMeTI* Workshop on "Membrane Applications in Agrofood" 2009, Cetraro (CS), Italy

E. Piacentini, L.Giorno, **R. Mazzei**, E. Drioli, New development for the controlled fabrication of microstructured multiphase bioreactor using membrane emulsification technology, *Symbiosis 2009*, Barcelona, Spain

R. Mazzei, L. Giorno, E. Piacentini, E. Drioli, Advances in Biocatalytic Membrane Reactors for the production of non commercially available pharmacologically active compounds from vegetal material, *Symbiosis 2009*, Barcelona, Spain

Rosalinda Mazzei, Lidietta Giorno, Silvia Mazzuca, Enrico Drioli, β-glucosidase heterogenization on polymeric membrane, Congresso Società botanica 2008, Italia

Rosalinda Mazzei, Lidietta Giorno, Kinetic study of β-glucosidase immobilized on polysulphone capillary membrane, Nanomemcourse, 2008, Patrasso, Grecia.

L. Giorno, E. Piacentini, **R. Mazzei**, F. Bazzarelli, E. Drioli, Membrane Emulsification Technology to Enhance Phase Transfer Biocatalyst Properties and Multiphase Membrane Reactor Performance, ICOM 2008, Honolulu/Kahuku Hawaii (July 12-18, 2008)

Rosalinda Mazzei, Lidietta Giorno, Silvia Mazzuca, Enrico Drioli Nanostructured microporous membranes functionalized with biomolecules, Nanomemcourse, 2007, Saragoza, Spagna.

L. Giorno, E. Piacentini, **R. Mazzei**, E. Drioli, R. Cassano, N. Picci, Multiphase enzyme membrane reactors for kinetic resolution of racemic esters: influence of OR-ester group lenght and oil/water interface, Permea 2007, Siófok, Hungary (2-6 September 2007)

L. Giorno, **R. Mazzei**, E. Piacentini, E. Drioli, Membranes in food industrial applications and research perspectives, Permea 2007, Siófok, Hungary (2-6 September 2007)

L. Giorno, E. Piacentini, **R. Mazzei**, F. Bazzarelli, E. Drioli, Biocatalytic membrane reactors: Advances in biotechnology and pharmaceutical applications, ICCMR8 2007, Kolkata (India)

L. Giorno, E. Piacentini, **R. Mazzei**, F. Bazzarelli, E. Drioli, Membrane emulsification and its application in production system optimization, China-EU Seminar on the Application of Membrane Technology & Cooperation Fair, Weihai (China) (July 8-12, 2007)

L. Giorno, E. Piacentini, **R. Mazzei**, E. Drioli, Distribution of phase transfer biocatalyst at the oil/water interface by membrane emulsifier and evaluation of enantiocatalytic performance, *Euromembrane 2006*, Giardini di Naxos Taormina (Messina), Italy.

Rosalinda Mazzei, Lidietta Giorno, Silvia Mazzuca, Antonia Spadafora, Emma Piacentini, Enrico Drioli, β-glucosidase separation from Olea europaea fruit and its use in membrane bioreactors for hydrolysis of oleuropein, Euromembrane 2006, Taormina (Messina) Italy.

Silvia Mazzuca, Lidietta Giorno, Antonia Spadafora, **Rosalinda Mazzei,** A new combined method to localize enzyme immobilized in polymeric membranes and evaluate its activity *in situ*. Euromembrane 2006, Taormina (Messina) Italy.

Education and Training

2009 – “Fundamentals of mass trasport in membrane processes” and “ Preparation of membranes for mass separation and energy conversion processes”, Institute on Membrane Technology , ITM-CNR, 15 Giugno-3 Luglio 2009, Prof. Heiner Strathmann.

2009- 9th International Conference on Catalytsis in Membrane Reactors ICCMR9, 28 Giugno-2 Luglio 2009, Lione, Francia.

2009-Participation to Euromembrane 2009, 6-10 Settembre 2009, Montpellier, Francia.

2009- Participation to IMeTi Workshop on “Membrane Applications in Agrofood” 18-20 Ottobre, Cetraro, Italia.

2008- English course “FIRST Certificate” with the result: level B2 upper

2008- Chemical Engineering Conference for Collaborative Research in Eastern Mediterranean Countries EMCC5 5th, Cetraro Italy 24-29 Maggio.

2008 –NanoMemCourse “EF2: Nanostructured material and membrane modelling and simulation”, Patras, Greece, 28 Giugno -27 Giugno 2008.

2006- Participation to congresso to ECI 2006”, Cetraro-Cosenza, Italy

2007- “Preparation, Characterization and Application of Phase Inversion Hollow Fiber Membranes” Istitute pn Membrane Technology ITM-CNR, Rende CS, prof. Heiner Strathmann.

2007- NanoMemCourse “Nanostructured Materials and Membrane synthesis and characterization” Saragoza Spain.

2007- English course “PET Certificate” , with the result: “Pass with merit”, level B1.

2006- Euromembrane 2006, Giardini Naxos, Taormina (Messina), Italy, 24-28 Settembre.