Università della Calabria
Facoltà di Ingegneria
Scuola di Dottorato "Pitagora" in Scienze Ingegneristiche
XXIII - I Ciclo (2007-2010)

SETTORE SCIENTIFICO DISCIPLINARE: CHIM/07 – Fondamenti Chimici delle Tecnologie

A Dissertation submitted in partial fulfilment of the requirements for the Doctorate of Philosophy degree on

DEVELOPMENT OF SUBMERGED BIOCATALYTIC MEMBRANE REACTORS FOR INNOVATIVE PRODUCTION SYSTEMS

Supervisor

Prof. Enrico DRIOLI

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Summary

Enzymatic membrane bioreactors (MBR) have been studied for very different applications since many years. Submerged MBR has also been successfully used for treatment of waste water. In the existing submerged configuration, the membrane works as the separation unit operation while the bioconversion is carried out by microorganisms suspended in the tank reactor. In the present work, a novel approach that combines the concept of biocatalytic membranes and submerged modules is proposed. Lipase enzyme from *Candida rugosa* has been immobilized in polyethersulphone hollow fibre (PES HF) membrane in order to develop a two separate phase biocatalytic submerged membrane reactor in which the membrane works as both catalytic and separation unit. The performance of the biphasic submerged enzymatic membrane reactor was evaluated based on the fatty acids extraction in the aqueous phase. The catalytic activity of lipase immobilized in the submerged system was studied at various operating conditions such as pH, T and fluid dynamics parameters. Furthermore, the submerged biocatalytic membrane reactor is intended for production of valuable components from waste streams, such as fried cooked oils. In this work, the proof of principle in using the submerged biocatalytic membrane reactor for the production of fatty acids from cooked vegetable oil (olive, corn, sunflower and soya oil) has been performed.

The overall thesis is organized in four different chapters. In the Chapter 1 the principles of membranes and membrane bioreactors are reported. The analysis of state of the art of membrane bioreactor and their application with particular emphasis in the

food application has been illustrated in Chapter 2. Chapter 3 describes the materials and methods used in the present work and the results are discussed in Chapter 4.

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This thesis is dedicated to

Tombaba, Munu Ma, my brother Goi, Misty, my family, Lidia & Prof. Drioli

Without all of your support, encouragement and inspiration it would never have been accomplished

Contents

Chapter 3: Material and Methods

Chapter 4: Results and Discussions

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Bagio, Τάσο, Θανάση, σας ευχαριστώ για αυτό που είστε. Τι.. να πω κι άλλα!;;

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Chapter 1

Introduction to membranes and membrane bioreactors

In this chapter the basic principles that govern membrane bioreactors and in particular biocatalytic membrane reactors are reported. First, the discussion will focus on the membrane, its characteristic, morphological structure, etc. Then, methods for enzyme immobilization in membranes and general aspects of biocatalytic membrane reactors kinetics will be discussed.

1.1 Membrane and its function

A precise and complete definition of a membrane that covers all its aspects is rather difficult, even when the discussion is limited to synthetic structures as in this outline. Various definitions have been reported in the literature, most of them focusing on the selective properties of the membrane. For example, Lonsdale defined a synthetic membrane as an interphase that separates two phases and restricts the transport of various components in a specific manner [Lonsdale 1982]. In new membrane processes, such as membrane contactors, the membrane is a non-selective barrier that separates and contacts the two adjacent phases. Therefore, in the most general sense we can define a membrane as a selective or non-selective barrier that separates and/or contacts two adjacent phases and allows or promotes the exchange of matter, energy, and information between the phases in a specific or non-specific manner. Separation of a mixture in a membrane process is the result of different transport rates of different components through the membrane. The transport rate of a component through a membrane is determined by driving forces such as concentration, pressure, temperature, and electrical potential gradients, and the concentration and mobility of the component in the membrane matrix.

A membrane can be homogeneous or heterogeneous, symmetric or asymmetric in structure. It may be solid or liquid and may consist of organic or inorganic materials. It may be neutral or it may carry positive or negative charges, or functional groups with specific binding or complexing abilities. Its thickness can be less than 100 nm to more than a millimeter. The electrical resistance may vary from more than 1,000,000 ohm cm^2 to less than one ohm cm^2. The term "membrane", therefore, includes a great variety of materials and structures, and a membrane can often be better described by its function rather than by its structure. Some materials, such as protective coatings, or packaging materials, show typical membrane properties, and are in fact membranes. Most materials functioning as membranes have one characteristic property in common: they restrict the passage of different components in a very specific manner. In some cases, a hydrophobic polymeric membrane may allow the passage of water as vapor phase but not as liquid phase, as for example in membrane distillation. In other cases, e.g. membrane contactors, the porous structure of the membrane material functions as a barrier that keeps in contact two adjacent phases between which a mass transfer occurs, and separates them at the same time. In both cases the membrane has no direct effect on the transport of various components and is non-selective. The concept of a selective and non-selective membrane is illustrated in **Fig 1.1** which shows in a) a membrane which is highly selective and capable of separating, e.g. two enantiomers and b) a membrane that acts as a barrier between two phases and avoids the mixing of the phases but has no effect on the transport of different components from one phase to the other.

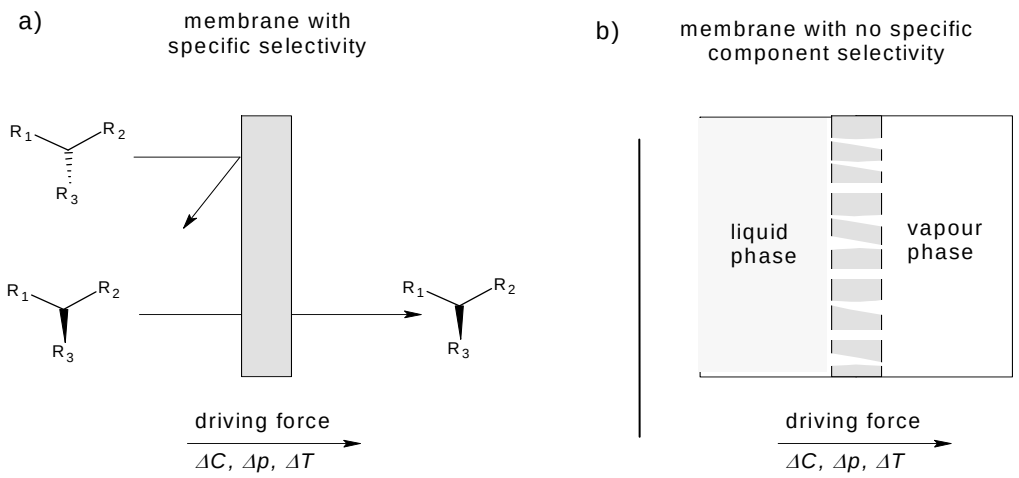


Fig. 1.1. a) Membrane which is selective for the transport of different components such as two enantiomers and
b) Membrane which separates a liquid and a vapour phase and allows passage of vapour molecules but is not selective for the transport of different components.

1.2 Different membrane processes

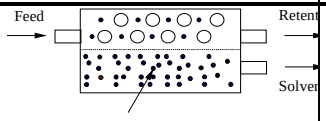
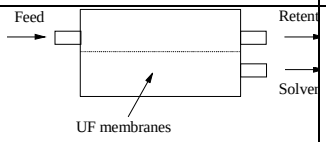
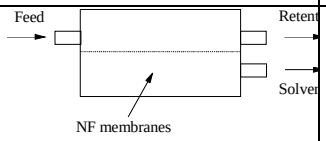
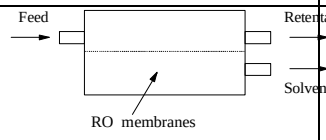
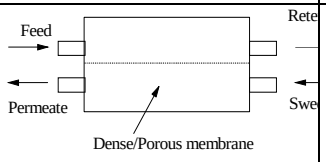
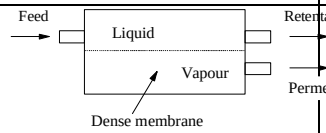
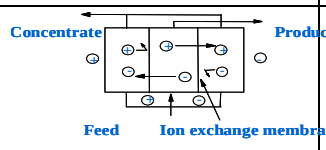
Membrane processes can be grouped according to the applied driving forces into: (1) hydrostatic pressure driven processes such as reverse osmosis, nano-, ultra- and microfiltration or gas separation, pervaporation;

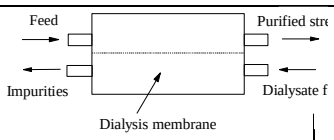
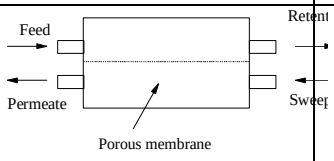
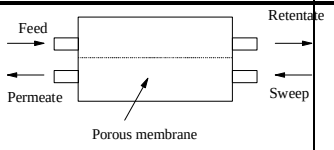
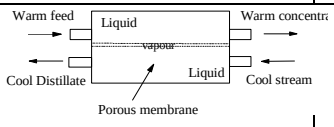
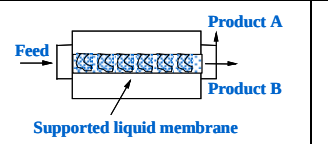
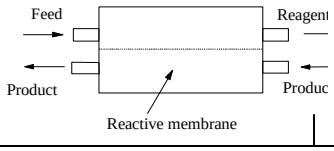
(2) concentration gradient or chemical potential driven processes such as dialysis, Donnan dialysis, pervaporation, and membrane contactors, such as membrane based solvent extraction, membrane scrubbers and strippers, osmotic distillation; (3) electrical potential driven processes such as electrodialysis; and

(4) temperature difference driven membrane processes such as membrane distillation.

The molecular mixture which will be separated is referred to as feed, the mixture containing the components retained by the membrane is called the retentate and the mixture composed of the components that have permeated the membrane is referred to as permeate (or filtrate in micro- and ultrafiltration). Table 1.1. lists the basic properties of membrane operations.

Table 1.1. Basic concepts of different membrane operations

Process	Concept	Driving Force	Mode of transport	Species Passed	Species Retained
Microfiltration (MF)		pressure difference 100 - 500 kPa	size exclusion convection	solvent (water) and dissolved solutes	suspended solids, fine particulars, some colloids
Ultrafitlration (UF)		pressure difference 100 - 800 kPa	size exclusion convection	solvent (water) and Low molecular weight solutes (<1000 Da)	macrosolutes and colloids
Nanofiltration (NF)		pressure difference 0.3 - 3 MPa	size exclusion solution diffusion Donnan exclusion	solvent (water), low molecular, weight solutes, monovalent ions	molecular weight compounds > 200 Da multivalent ions
Reverse Osmosis (RO)		pressure difference 1 - 10 MPa	solution diffution mechanism	solvent (water)	dissolved and suspended solids
Gas Separation (GS)		pressure Difference 0.1 – 10 MPa	solution diffusion mechanism	gas molecules having Low molecular weight or high solubility-diffusivity	gas molecules having high molecular weight or low solubility-diffusivity
Pervaporation (PV)		chemical potential or concentration difference	solution diffusion mechanism	high permeable solute or solvents	less permeable solute or solvents
Electrodialysis (ED)		electrical potential difference 1 – 2 V / cell pair	Donnan exclusion	solutes (ions) Small quantity of solvent	non-ionic and macromolecular species

Dialysis (D)		concentration difference	diffusion	solute (ions and low MW organics) Small solvent quantity	dissolved and suspended solids with MW > 1000 Da
Membrane contactors (MC)		chemical potential, concentration difference; temperature difference	diffusion	compounds soluble in the extraction solvent; volatiles	compounds non soluble in the extraction solvent; Non volatiles
Membrane based solvent extraction (MBSX)		chemical potential or Concentration difference	diffusion partition	compounds soluble in the extraction solvent	compounds non soluble in the extraction solvent
Membrane Distillation (MD)		temperature difference	diffusion	volatiles	non volatiles
Supported liquid membranes (SLM)		concentration difference	diffusion	ions, low MW organics	ions, less permeable organics
Membrane reactors (MR)		various	various	permeable product	non permeable reagents

1.3 The membrane transport mechanisms

The mechanism by which certain components are transported through a membrane can also be very different. In some membranes, for example, the transport is based on viscous flow of a mixture through individual pores in the membrane caused by a hydrostatic pressure difference between the two phases separated by the membrane. This type of transport is referred to as *viscous flow*. The components that permeate through the membrane are transported by convective flow through micropores under a gradient pressure as driving force and the

separation occurs because of size exclusion as indicated in **Fig 1.2** a). Darcy's Law describes this type of transport. It is the dominant form of mass transport in micro- and ultrafiltration but also occurs in other membrane processes.

If the transport through a membrane is based on the solution and diffusion of individual molecules in the non-porous membrane matrix due to a concentration or chemical potential gradient the transport is referred to as *diffusion*. The separation occurs because of different solubility and diffusivity of components into the membrane material as indicated in Figure 1.2 b). The Fick's Law describes this type of transport. The diffusion of molecules through homogeneous dense membranes occurs through the free volume elements, or empty spaces between polymer chains caused by thermal motion of the polymer molecules, which fluctuate in position and volume on the same time scale as the molecule permeates. The transition between fluctuating free volumes and individual permanent pores is controversial. In general it is considered in the range of 5-10 Å in diameter.

This form of mass transport is dominant in reverse osmosis, gas separation, pervaporation or dialysis but it may occur in other processes too. If an electrical potential gradient across the membrane is applied to achieve the desired transport of certain components through the membrane the transport is referred to as *migration*. Migration occurs in electrodialysis and related processes and is limited to the transport of components carrying electrical charges such as ions.

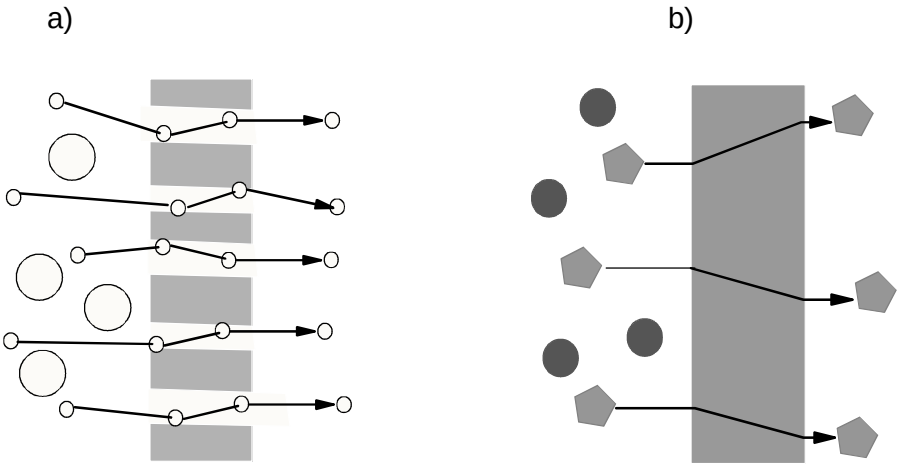


Fig. 1.2. a) The sieving mechanism of a porous membrane and
b) The solution diffusion mechanism in a non-porous membrane.

1.4 Materials and structures of synthetic membranes

Synthetic membranes show a large variety in their physical structure and the materials they are made from. Based on their structure they can be classified in four groups:

- porous membranes,
- homogeneous solid membranes,
- solid membranes carrying electrical charges,
- liquid or solid films containing selective carriers.

Furthermore, the structure of membranes may be symmetric, i.e. the structure is identical over the entire cross-section of the membrane or it may be asymmetric, i.e. the structure varies over the cross-section of the membrane.

The materials used for the preparation of membranes can be polymers, ceramics, glass, metals or liquids. The materials may be neutral or carry electrical charges, i.e. fixed ions. The membrane conformation can be flat, tubular or a hollow fiber. The schematic drawing of Fig. 1.3 illustrates the morphology, materials and configuration of some technically relevant synthetic membranes.

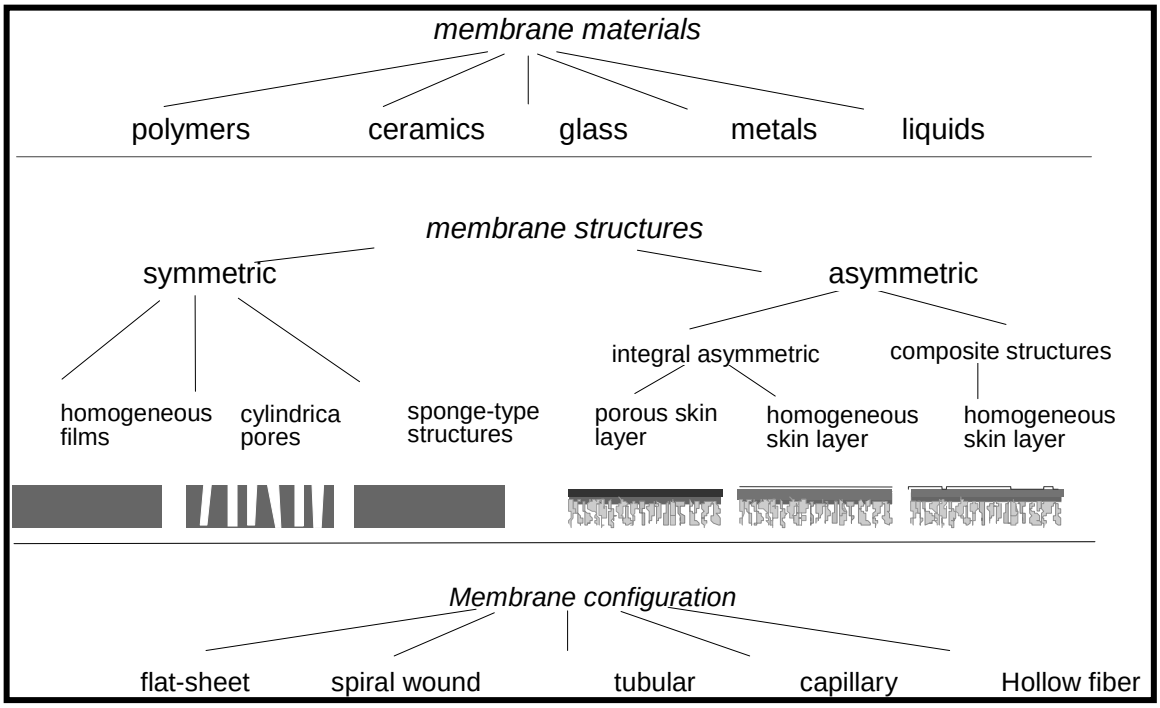


Fig. 1.3 Schematic drawing illustrating the various materials, structures and configuration of technically relevant synthetic membranes.

1.4.1 Symmetric and asymmetric membranes

As indicated earlier synthetic membranes may have a symmetric or an asymmetric structure. In a symmetric membrane the structure and the transport properties are identical over the entire cross-section and the thickness of the entire membrane determines the flux. Symmetric membranes are used today mainly in dialysis and electrodialysis. In asymmetric membranes structural as well as transport properties vary over the membrane cross-section. An asymmetric membrane consists of a 0.1 to 1 µm thick "skin" layer on a highly porous 100 to 200 µm thick substructure. The skin represents the actual selective barrier of the asymmetric membrane. Its separation characteristics are determined by the nature of the material or the size of pores in the skin-layer. The mass flux is determined mainly by the "skin" thickness. The porous

sub-layer serves only as a support for the mostly thin and fragile skin and has little effect on the separation characteristics or the mass transfer rate of the membrane **Fig. 1.4**. Asymmetric membranes are used primarily in pressure driven membrane processes such as reverse osmosis, ultrafiltration, or gas and vapor separation, since here the unique properties of asymmetric membranes, i.e. high fluxes and good mechanical stability can best be utilized. Two techniques are used to prepare asymmetric membranes: one utilizes the phase inversion process which leads to an integral structure with the skin and the support structure made from the same material in a single process [Kesting, 1971], and the other resembles a composite structure where a thin barrier layer is deposited on a porous substructure in a two step process [Cadotte et al., 1981]. In this case barrier and support structures are generally made from different materials.

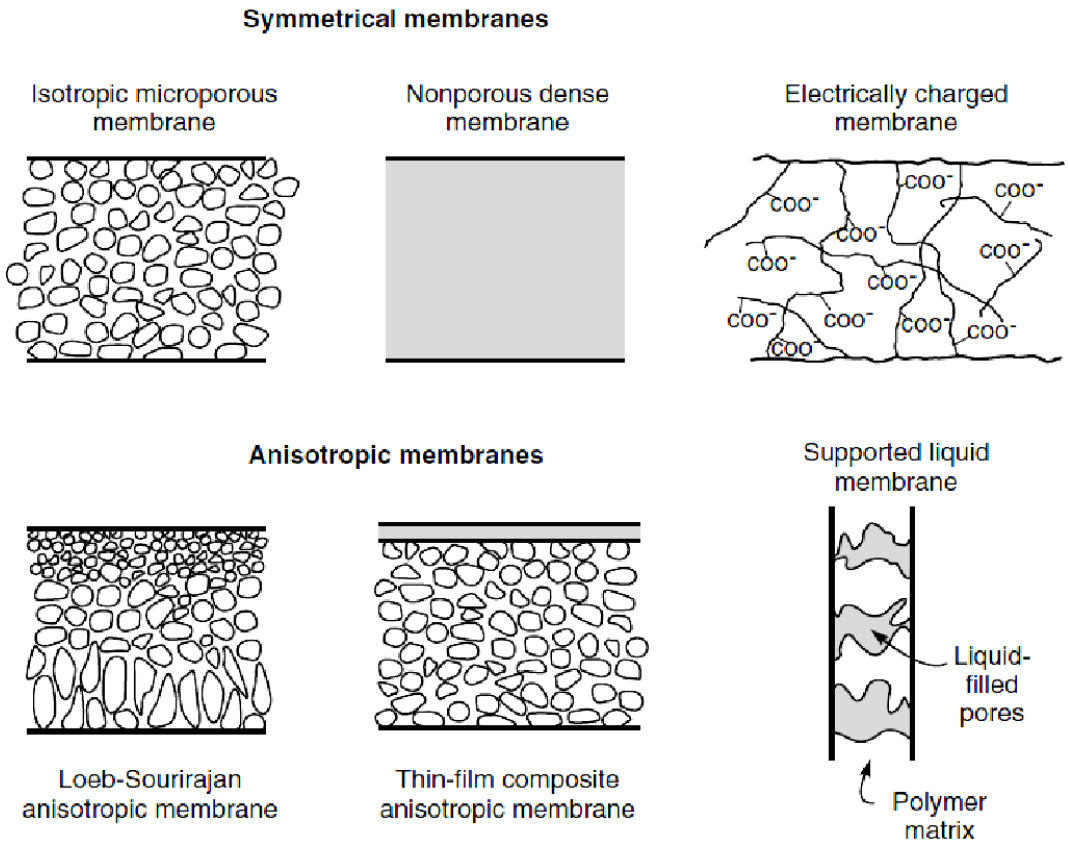
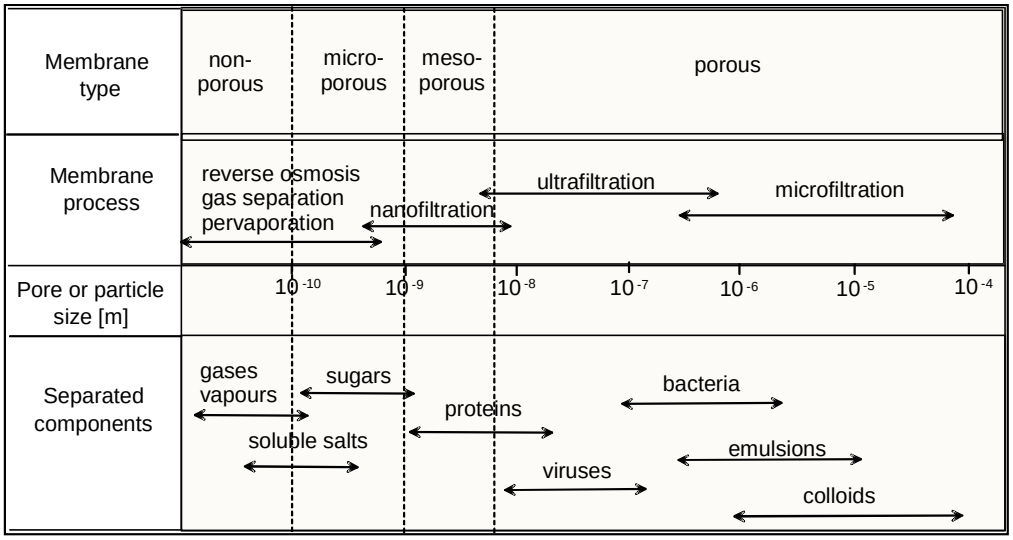


Fig 1.4 : Schematic diagrams of the principal types of membranes

1.4.2 Porous membranes

A porous structure represents a very simple form of a membrane, which closely resembles the conventional fiber filter as far as the mode of separation is concerned. These membranes consist of a solid matrix with defined holes or pores which have diameters ranging from less than 1 nm to more than 10 µm. The macromolecular size of the species to be separated plays an important role in determining the pore size of the membrane to be utilized and the related membrane process. Porous membranes with average pore diameters larger than 50 nm are classified as macroporous, and those with average pore diameters in the intermediate range between 2 and 50 nm are

classified as mesoporous. Membranes with average pore diameters between 0.1 and 2 nm are classified as microporous. Dense membranes have no individual permanent pores, but the separation occurs through fluctuating free volumes. The schematic representation of such classification is illustrated in Fig. 1.5 (a,b).



a

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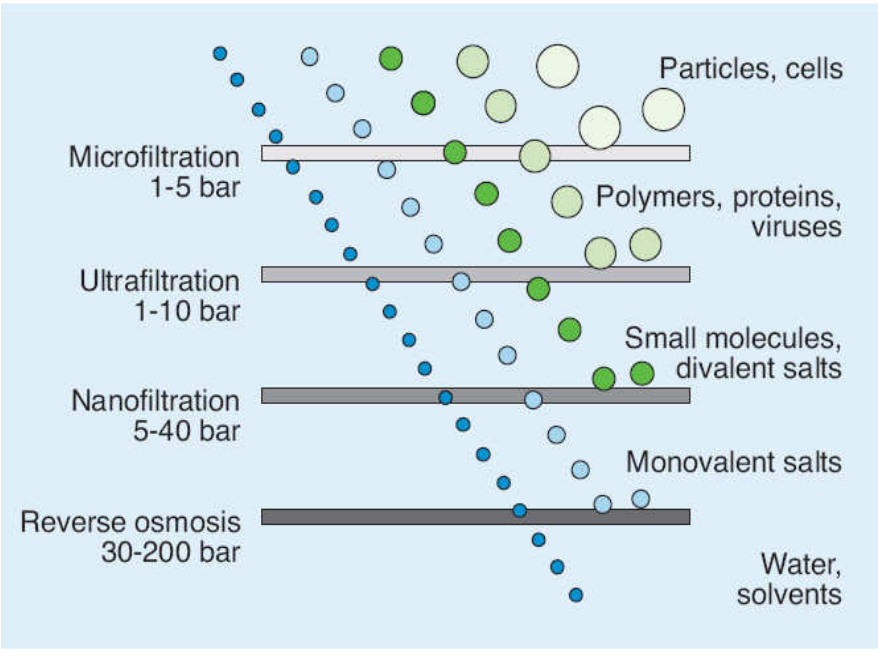


b

Fig. 1.5 a.b.. Schematic classification of membranes, related processes and separated components

In pressure driven membrane processes the separation of the various components is achieved by a sieving mechanism with the pore diameters and the particle sizes being the determining parameters. In thermally driven membrane processes the separation is based on the principle of phase equilibrium and the non-wet ability of membrane pores is the determining parameter. Porous membranes can be made from various materials such as ceramics, graphite, metal or metal oxides, and various polymers. Their structure may be symmetric, i.e. the pore diameters do not vary over the membrane cross-section, or they can be asymmetric, i.e. the pore diameters increase from one side of the membrane to the other typically by a factor of 10 to 1000. The techniques for the preparation of porous membranes can be rather different and include simple pressing and sintering of polymer or ceramic powders, irradiation and leaching of templates as well as phase inversion and polymer precipitation procedures or sol-gel conversion techniques. Porous membranes are used to separate components that differ markedly in size or molecular weight in processes such as micro- and ultrafiltration or dialysis [Cheryan, 1998].

1.4.3 Homogeneous dense membranes

A homogeneous membrane is merely a dense film through which a mixture of molecules is transported by a pressure, a concentration, or an electrical potential gradient. The separation of the various components of a mixture is directly related to their transport rates within the membrane phase, which is determined by their diffusivities and concentrations in the membrane matrix. Therefore, homogeneous membranes are referred to as solution-diffusion type membranes [Merten, 1966]. They

can be prepared from polymers, metals, metal alloys or, in some cases, ceramics which may also carry positive or negative electrical charges. Since the mass transport in homogeneous membranes is based on diffusion their permeabilities are rather low. Homogeneous membranes are used mainly to separate components which are similar in size but have different chemical nature in processes such as reverse osmosis, gas and vapor separation, and pervaporation [Raymond et al., 1992]. In these processes asymmetric membrane structures are used which consist of a thin homogeneous skin supported by a porous substructure. Some of the well known polymer used to prepare different membranes has been tabled in table 1.2.

Table 1.2 : Polymeric membrane materials (Judd, 2006; Mulder, 1996)

Material	Abbreviation	Applications
Polyvinylidene difluoride	PVDF	MF, UF
Polyethylsulphone	PES	UF, RO
Polyethylene	PE	MF, UF
Polypropylene	PP	MF, UF

1.5 System configuration

The membrane step can be implemented into an MBR system in two ways: sidestream or immersed. In sidestream configuration the membranes are placed externally from the bioreactor, schematically presented in **Fig. 1.6**. The first generation MBRs were designed according to the sidestream configuration. Fouling was traditionally prevented by applying a high liquid cross flow through the membrane tubes.

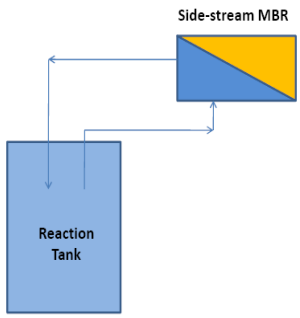


Fig 1.6: Side stream configuration

Applying a high cross flow velocity makes the membranes less sensitive to fouling and thus allows for employing a relatively high TMP or flux. The main disadvantage of the traditional sidestream configuration is the high energy consumption required for the cross flow. In the immersed or internal configuration the membrane modules are submerged directly into the activated sludge, either in a separate membrane tank or directly in the bioreactor. The immersed MBR configuration is schematically presented in **Fig. 1.7**. Nowadays the membranes are more commonly placed in a separate membrane tank; in the first place this allows better control over the filtration process and besides this the wastewater undergoes superior biological treatment before reaching the membrane. The disadvantage of a separate membrane tank is the higher energy consumption for recirculation and aeration.

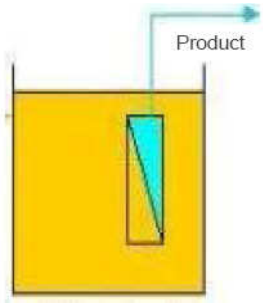


Fig 1.7: Submerged configuration

Within immersed MBR systems two different membrane configurations are applied: hollow fibre and flat sheet membranes. Some specific characteristics of the traditional sidestream and the immersed MBR configuration are summarized in **Table 1.3** (Evenblij 2006; Judd 2006).

Table 1.3: Summary of sidestream and immersed MBR characteristics

Characteristic	Sidestream (liquid crossflow)	Immersed (coarse bubble aeration)
Membrane placement	Outside reactor	In bioreactor
TMP	2 – 6 Bar	0.2 – 0.5 Bar
Filtration type	Inside-out	Outside-in
Flux	50 – 100 L/m2·h	20 – 40 L/m2·h
Energy consumption	2 – 5 kWh/m3	0.2 – 0.5 kWh/m3
Crossflow speed	1 – 6 m/s	0.5 m/s

1.6. Enzyme

Different enzymes have been already applied in many different biocatalytic applications. It is well understood that the enzyme has some specific advantages over inorganic, metal-derived or chemical catalysts. The interest in lipase oriented research is increasing over the past decades. This is primarily due to their ability to utilize a wide spectrum of substrates, high stability towards extremes of temperature, pH and organic solvents, and chemo-, regio- and enantioselectivity. Products with high purity and high added value can be obtained. Moreover, the use of enzyme decreases the side reactions and simplifies post-reaction separation problems (Pandey et al., 1999). Activation energy of this enzyme is low, thus, require mild reaction conditions of pH and temperature, which lead to decrease in energy consumption and less thermal damage to reactants and products (Balcao et al., 1996). Lipases (EC.3.1.1.3, triacylglycerol acylhydrolyse) have been used for many years. Therefore, customization of lipases by chemical and physical modifications has been attempted to

improve their catalytic properties in hydrolysis and synthesis involving aqueous, non-aqueous and organic solvents. In addition, the cost of enzyme can be reduced by the application of molecular biological tools, this may allow the production of lipases in large quantities and with genetically enhanced properties (Villeneuve et al., 2000). These properties allow them to catalyze reactions with reduced side products, lowered waste treatment costs and under conditions of mild temperature and pressure. Secondly, they are widely distributed among the animals, plants and microorganisms and their activity is greatest against water-insoluble substrates and enhanced at the substrate (oil)-water interface; that is, they exhibit .interfacial activation. Thus, maximum activities are obtained in emulsion systems where high surface areas of the substrate can be obtained. Lipases are active not only in normal phase emulsions where the substrate is emulsified into an aqueous system (oil-in-water), but they are also active, often more active, in invert (water-in-oil) emulsions and in reverse micelle systems containing an organic solvent solution of the substrate. Thus, it is not surprising that lipases, for many years, served as models for studying the regulation of interfacial, enzyme-catalyzed reactions. Final and the most important reason is linked to the enzyme's medical relevance, particularly to atherosclerosis and hyperlipidemia, and its importance in regulation and metabolism, since products of lipolysis such as free fatty acids and diacylglycerols play many critical roles, especially as mediators in cell activation and signal transduction. Important areas of industrial applications of microbial lipases has summarized in **Table 1.4**:

Table 1.4 : Industrial applications of microbial lipases

INDUSTRY	EFFECT	PRODUCT
Bakery	Flavour improvement and shelf life prolongation	Bakery products
Beverages	Improved aroma	Beverages
Chemical	Enantioselectivity	Chiral building blocks and chemicals
Cleaning	Synthesis Hydrolysis	Chemicals Removal of cleaning agents like surfactants
Cosmetics	Synthesis	Emulsifiers, moisturising agents
Dairy	Hydrolysis of milk fat Cheese ripening Modification of butter fat	Flavour agents Cheese Butter
Fats and oils	Transesterification Hydrolysis	Cocoa butter, margarine Fatty acids, glycerol, mono- and diglycerides
Food dressing	Quality improvement	Mayonnaise, dressings and whippings
Health food	Transesterification	Health food
Leather	Hydrolysis	Leather products
Meat and fish	Flavour development and fat removal	Meat and fish products
Paper	Hydrolysis	Paper products
Pharmaceutical	Transesterification Hydrolysis	Specially lipids Digestive aids

(http://www.iisc.ernet.in/~currsci/july10/articles18.htm).

1.7 Different Immobilization Technique

The catalyst/enzyme 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. 1.8** and detail discussion later on.

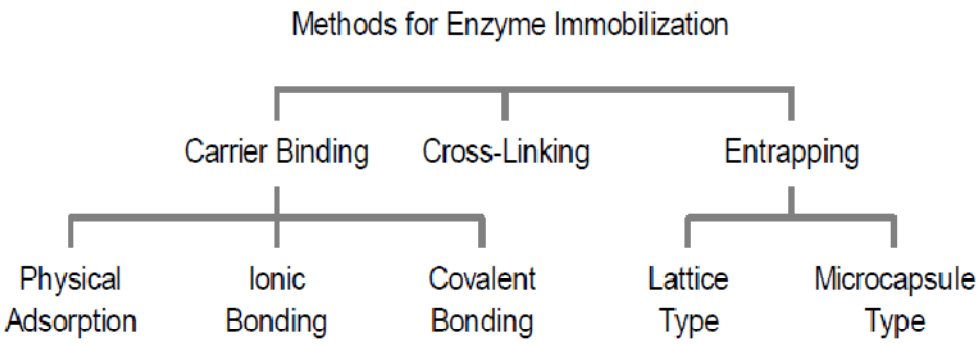


Fig 1.8 : Classification of enzyme immobilization techniques

1.7.1. Entrapment

The entrapment method **(Fig: 1.9)** 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 fibre 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). The transport of the substrate through the enzyme-

loaded membrane is another important parameter as well as the residence-time (Strathmann, 2006).

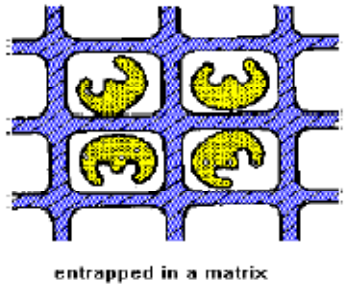


Fig. 1.9. : Schematic illustration of entrapment methods

1.7.2. Gelification

The gelification (**Fig 1.10**) 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).

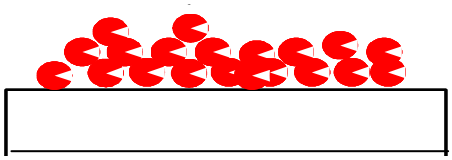


Fig 1.10: Gelification method

1.8 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.

1.8.1. Physical adsorption

Physical adsorption (**Fig. 1.11**) 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 (Goel, 1994).

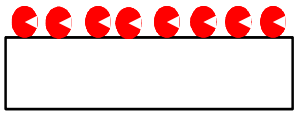


Fig 1.11: Physical Adsorption method

1.8.2. Ionic Binding

The ionic binding method (**Fig 1.12**) relies on the ionic binding of the enzyme protein to water-insoluble carriers containing ion-exchange residues. Polysaccharides and synthetic polymers having ion-exchange centers are usually used for carriers. The binding of enzyme to the carrier is easily carried out, and the conditions are much

milder than those needed for the covalent binding method. Hence, the ionic binding method causes little changes in the conformation and the active site of the enzyme, and so yields immobilized enzymes with high activity in most cases. As the binding forces between enzyme protein and carriers are less strong than in covalent binding; leakage of enzyme from the carrier may occur in substrate solutions of high ionic strength or upon variation of pH. The main difference between ionic binding and physical adsorption is that the enzyme to carrier linkages is much stronger for ionic binding although less strong than in covalent binding.

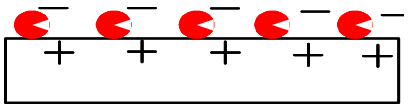


Fig 1.12 : The ionic binding method

1.8.3. Covalent Binding

The covalent binding method (**Fig: 1.13**) is based on the binding of enzymes and membrane by covalent bonds. The functional groups that take part in this binding of enzyme to membrane can be amino, carboxyl, sulfhydryl, hydroxyl, imidazole or phenolic groups which are not essential for the catalytic activity. In order to protect the active site, immobilization can be carried out in the presence of its substrate or a competitive inhibitor. Activity of the covalent bonded enzyme depends on the size and shape of carrier material, nature of the coupling method, composition of the carrier material and specific conditions during coupling. The main advantage of the covalent attachment is that such an immobilization is very stable. Unlike physical adsorption, the binding force between enzyme and carrier is so strong that no leakage of the

enzymes occurs, even in the presence of substrate or solution of high ionic strength. Moreover, the obtained immobilized enzymes are usually very stable and resistant to extreme conditions (pH range, temperature). Finally, a large number of different supports and methods to activate them are available. However, experimental procedures are obviously more difficult to carry out than for physical adsorption. The 3-D structure of the protein is considerably modified after the attachment to the support. This modification may lead to a loss of the initial activity of the biocatalyst (Villeneuve et al., 2000). Examples of derivatized lipases obtained through this procedure include PEG modified lipase, fatty acid-modified lipase, amidinated lipase, and detergent-modified lipase (Villeneuve et al., 2000). (Xie, 1999) (Charcosset 2006).

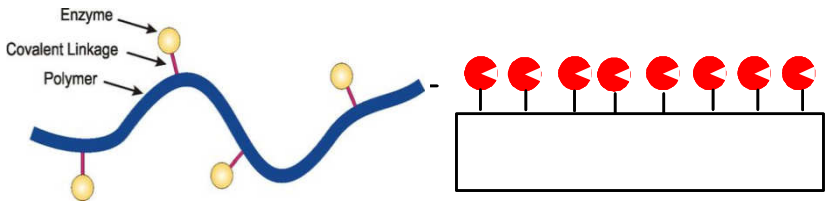


Fig 1.13: Covalent binding method

1.8.4. 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 (**Fig 1.14**) 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.

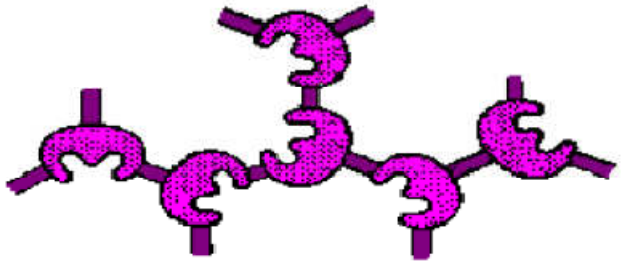


Fig 1.14 : Cross-linking of enzyme

1.9 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.

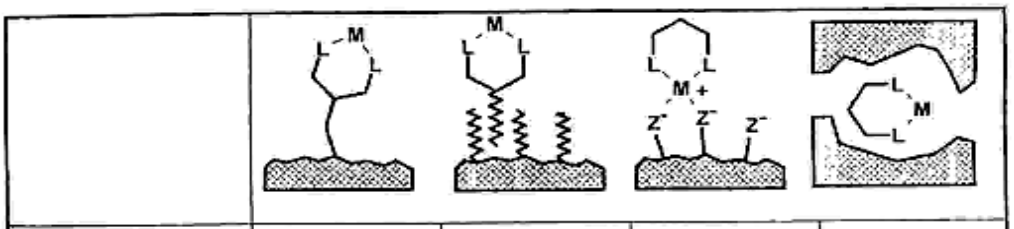
In literature there are various routes to carry enzyme immobilization creating a bound on supports, the principal strategies are based on chemical grafting or molecular recognition on porous supports.

The 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 absorb 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. The different drawbacks of enzyme immobilization is described in the following picture (Fig 1.15)



Immobilization method	covalent binding	adsorption	ion pair formation	entrapment or 'ship in a bottle'
Applicability	broad	restricted	restricted	restricted
Problems	preparation	competition with solvents, substrates	competition with ionic substrates, salts	size of substrate, diffusion

Fig 1.15 : Drawbacks of enzyme immobilization techniques

1.10 Membrane Reactor

Membrane reactors are systems where a chemical or biochemical conversion is combined with a membrane separation process. The two main areas of membrane reactors are identified by the type of catalyst used, traditional chemical catalysts or catalysts of biological origin, which also dictates the operating conditions. Hence, membrane reactors (mainly inorganic) working at high temperature (100<T< 600 °C) or membrane bioreactors working at low temperature (< 100 °C) are known. Both types of reactors have two main configurations. In one case, the membrane is catalytically inert; it does not participate in the reaction directly; it simply acts as a barrier to reagents allowing selective separation of the product(s) or intermediate(s). This type of system is known with the general term “membrane reactor”. In the other

type of reactor, besides the ability to separate, the membrane is also catalytic, it contains the (bio)catalyst and participate directly in the reaction; hence, the system is called “catalytic membrane reactor. The details are discussed below.

1.10.1 Membrane bioreactors

Membrane bioreactors 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.

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 exhibits 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. 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. In a particular case of this membrane reactor is the continuous membrane fermentator or cell-recycle membrane fermentor 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 (**Fig 1.16**) and is not lost in the effluent stream, this system is called enzymatic membrane reactor (EMR).

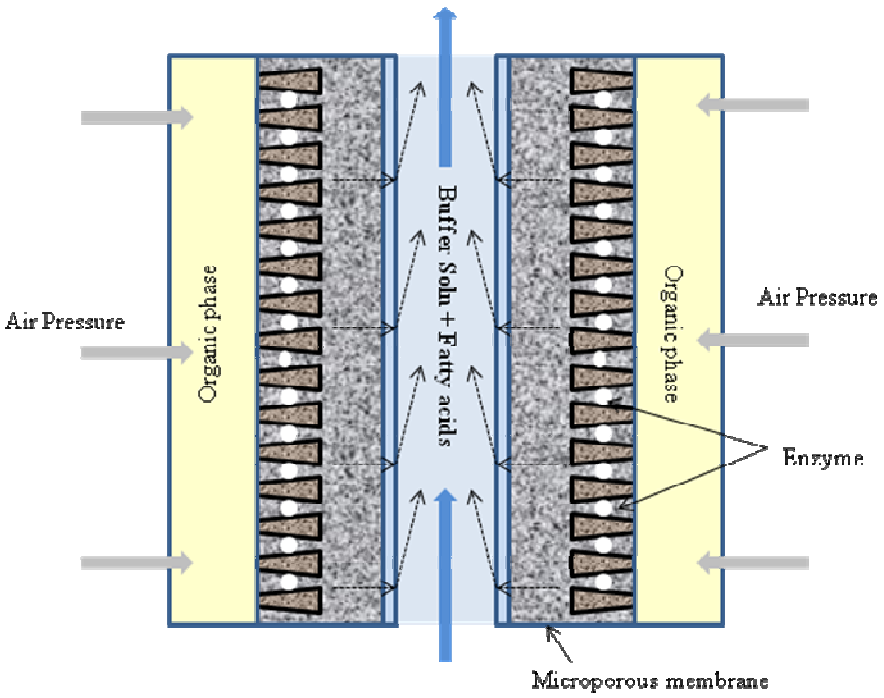


Fig 1.16 : Enzymatic Membrane Reactor where enzyme trapped inside the porous media of membrane which acts as a reactor as well as separator unit.

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).

1.10.2 Biocatalytic membrane reactors

In this kind of reactor in addition to separation action membrane is also a catalytic unit. The different kind of biocatalytic 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 properties of the species in solution and of the membrane itself. 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.

1.11 Principles of biocatalytic membrane reactor

After the immobilization technique to characterize the bioreactor, in terms of catalytic aspects, different concepts have to be considered. 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 is calculated as follows (Eq 1.1):

$$Conversion = \frac{C_r - C_p}{C_r} \quad \text{……..} \qquad \text{(Eq1.1)}$$

Where C_r is the concentration of the substrate in the retentate solution and C_p in the permeate solution. Another important parameter for bioreactor characterization in terms of catalytic aspects is the calculation of the reaction rate. The reaction rate equation for a membrane reactor can be derived by mass balance equation at steady-state.

In the case of a continuous stirred tank reactor, i.e. where the accumulation term is zero, the steady state mass balance equation is:

$$FC_f - FC_p + v_r V = 0 \quad \text{……..……(Eq 1.2)}$$

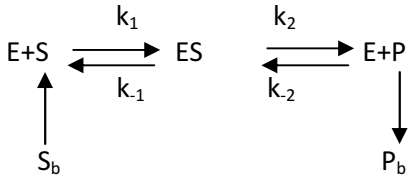
resolving for v_r the following is obtained:

$$v_r = \frac{F(C_f - C_p)}{V} \quad \text{……..…… (Eq 1.3)}$$

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 (i.e. the membrane void volume when the enzyme is immobilized within the membrane) (cubic centimetre).

For the various types of 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 etc.

To calculate the reaction kinetics for a biocatalytic membrane reactor it is necessary to consider the chemical equation that describes an enzymatic reaction taking into the account also the mass transfer properties, that is:



Eq. 1.4

Where Sb is the substrate in the bulk solution, which has to reach the immobilized enzyme, and Pb is the product in the bulk solution.

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

$$V_0 = \frac{V_{\max}[S]}{K_m + [S]} \quad \text{(Eq 1.5)}$$

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}:

$$\frac{1}{V_0} = \frac{K_m}{V_{\max}}\frac{1}{S} + \frac{1}{V_{\max}} \quad \text{(Eq 1.6)}$$

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 $\frac{K_m}{V_{\max}}$ which is considered as the reaction rate of the reaction.

In immobilized systems, in addition to the reaction kinetics properties, mass transfer properties have to be taken into the account in order to evaluate the overall reactor performance. In the following paragraphs the cases where the enzyme is immobilized on the membrane surface or within the membrane matrix are illustrated.

1.12 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:

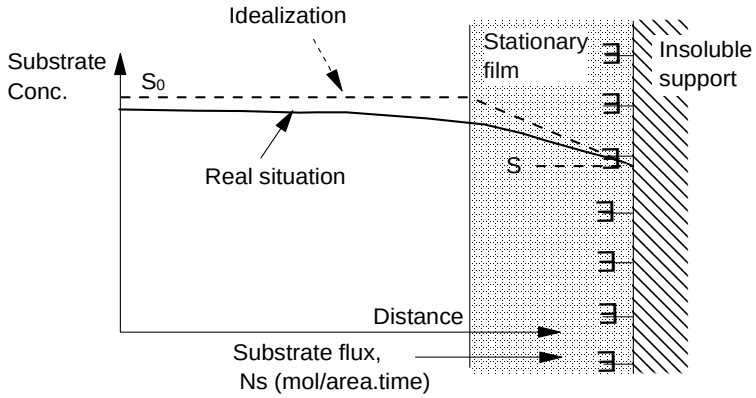


Fig. 1.17 Schematic representation of transport for catalyst immobilized on the surface of insoluble support

$$J_s = k_s(S_0 - S) = \frac{V_{\max}[S]}{K_m + [S]} \quad \text{……..……..(Eq 1.7)}$$

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 which can be written in the form of:

$$Da = \frac{V_{\max}}{k_s S_0} \quad \text{……..…….(Eq: 1.8)}$$

Two different phenomenon can be derived from this equation. Those are :

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:

$$V_{kin} = \frac{V_{\max}[S]_b}{K_m + [S]_b} \quad \text{……..(Eq: 1.9)}$$

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:

$$\eta = \frac{Observedreactiorate}{Rateobservedwithoutmasstrasferresistance}$$

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 (Drioli Giorno 1999, and strathmann 2006)

1.13 Enzyme immobilized into the porous matrix of membranes

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:

$$D_{eff} = D_{S_0} \frac{\varepsilon_p}{\tau} \frac{K_p}{K_r} \quad \text{……(Eq:1.10)}$$

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.

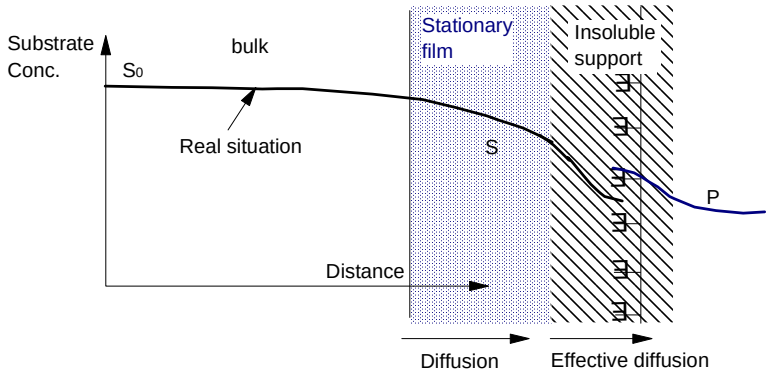


Fig 1.18: Concentration profiles through catalytic porous matrix

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:

$$D_{eff}\frac{d^2[S]}{dx^2}-\frac{V_{\max}[S]}{K_m+[S]}=0 \qquad \text{.....(Eq 1.11)}$$

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:

$$\phi = L\left(\frac{V_{\max}}{D_{eff}K_m}\right)^{1/2} \qquad \text{.....(Eq:1.12)}$$

which has the meaning of a reaction rate [(Drioli Giorno 1999, and strathmann 2006).

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Chapter 2

Membrane bioreactors research development and applications

This chapter illustrates the research development in the field of membrane bioreactors and their application in various fields. The chapter starts with an overview of the distribution of research activities over the years in various countries of the globe. The last part of the chapter focuses on the discussion of MBR in food applications.

2. 1 Chronological distribution of MBR research

Although the MBR concept, coupling of biological treatment process and membrane technology, was put forward over 30 years ago, there were very few journal papers published and available until the early and middle of the 1990s as presented in **Fig. 2.1** but the MBR research began in late 1960s. We will discuss about the past 20 years MBR research in different part of the world since we can only avail the online database of some of the popular scientific journals for our analysis. The research mainly focused on biomass separation MBR (BSMBR) with limited studies on extractive MBR (EMBR) and membrane aeration bioreactor (MABR), etc. The previous research contents included MBR configuration and type, membrane material and module, membrane fouling and control, characteristics of various wastewater treatment and other aspects like gas removal and microbial fermentation, etc. Two online databases including Web of Science and Elsevier, were searched for article papers written about different MBR research application for our analysis with some of the selective key words like MBRs, Bioreactor etc. After the middle of 1990s, there was a near linear increase in research outputs in different areas of life. And, a significant jump is

observed between 2001 and 2002. This will also result in wider acceptance of MBR technology as a mainstream alternative for water and wastewater treatment by both the scientific community and end-users (Visvanathan 2000, Sulton 2003) as well as other application. From the analysis it has been seen that over 75% of all studies on MBRs were conducted mainly at the following eight countries UK, USA, Japan, France, China, South Korea, Germany and Canada (**Fig. 2.2**) with side stream and submerged (**Fig 2.3**) configuration.

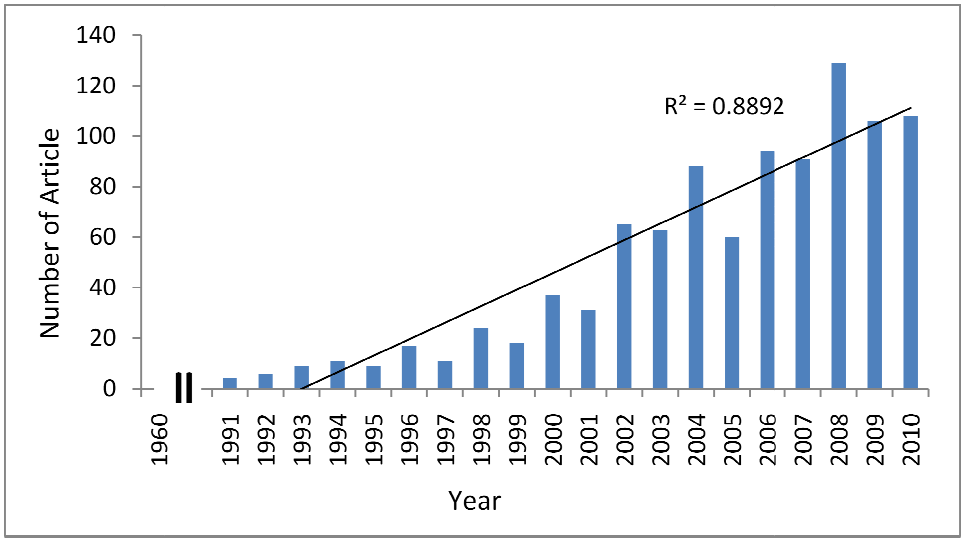

Fig. 2.1: Chronological distribution of worldwide, peer-reviewed journal articles involving studies on MBRs.

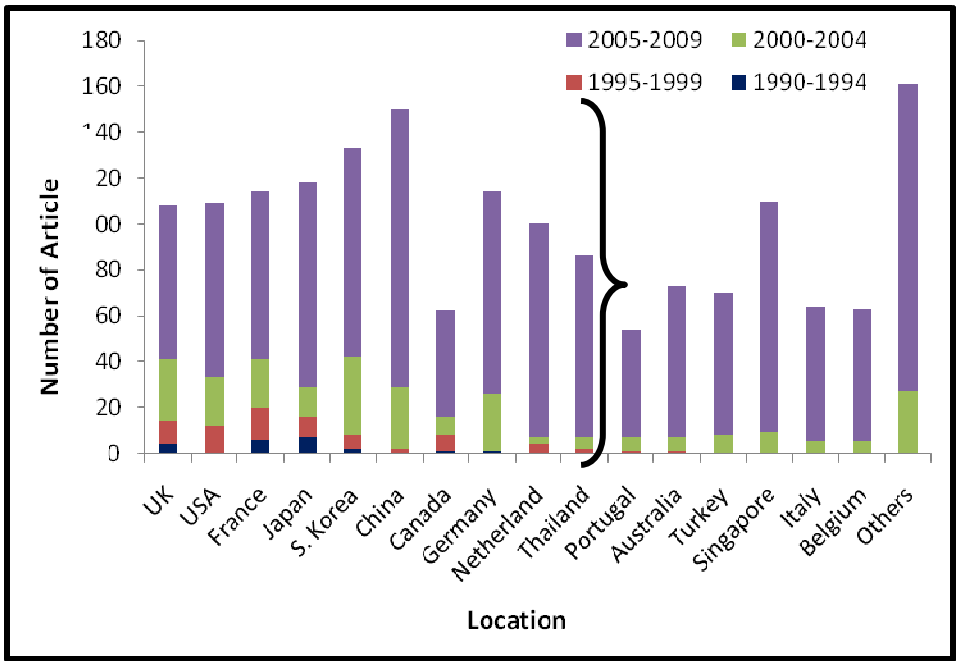

Fig. 2.2: Distribution of studies on MBRs in different countries

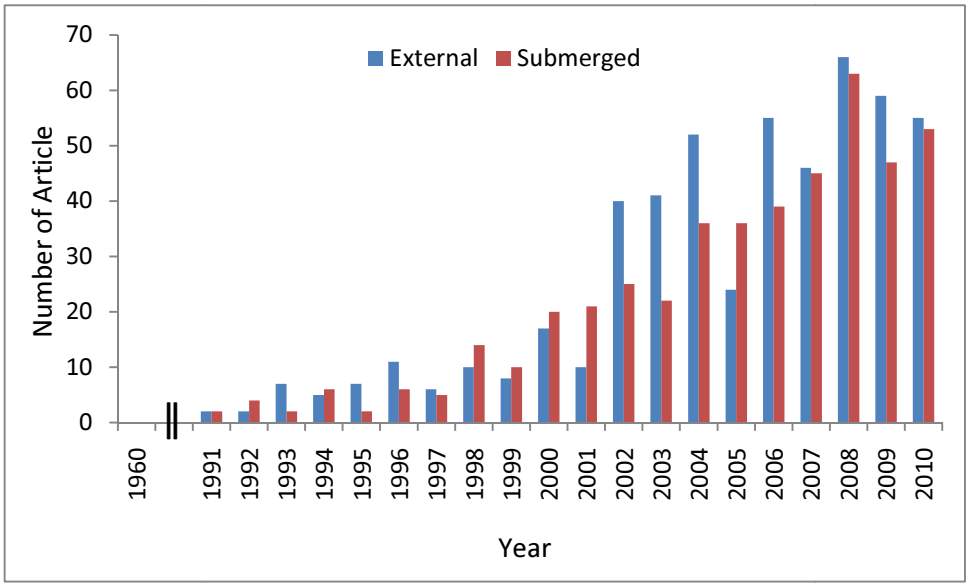

Fig. 2.3: Chronological distribution of studies on submerged and external MBRs.

This work could be the first of its kind to immobilize enzyme inside the shell side of the membrane and used as a reactor as well as separator to get high value product in submerged condition. The other potential application of membrane bioreactor has been described below.

2.2 Areas of MBR application

Membrane reactors using biological catalysts can be used in production, processing and different treatment operations. The recent trend towards environmentally friendly technologies makes these membrane reactors particularly attractive because they do not require additives, are able to function at moderate temperature, pressure, and reduce the formation of by-products.

Promising areas of expansion include food-processing wastewater, slaughterhouse wastewater and landfill leachates (Mazzei et al. 2010,Cicek 2003, Xing 2000, Ahn 2002) cheese whey treatment , wine industry, fruit juice preparation and concentration, agro food industry, oil, fats etc. Since the 1950s, when protease was first immobilized on diazotized polystyrene, many enzymes and microorganisms have been used in membrane reactors to catalyse bioconversions (Alvarez- Vazquez 2004). The different application of enzyme is shown in Table 2.1 below as a reference to understand the trend of research with enzyme as a biocatalyst.

Table 2.1: Applications of enzymes in different industrial production (12)

Type of industry	Enzyme	Application
Detergent	Proteases	To remove organic stains
	Lipases	To remove greasy stains
	Amylases	To remove residues of starchy foods
	Cellulases	To restore a smooth surface to the fibre and restore the garment to its original colours
Food	Proteases and lipases	To intensify flavour and accelerate the aging process
	Lactases	To produce low-lactose milk and related products for special dietary requirements
Wine	β-Glucanase	To help the clarification process
	Cellulase	To aid the breakdown of cell walls
	Cellulase and pectinase	To improve clarification and storage stability
Fruit juices	Pectinases	To improve fruit-juice extraction and reduce juice viscosity
	Cellulase	To improve juice yield and colour of juice
Oils and fats	Lipases	The industrial hydrolysis of fats and oils or the production of fatty acids, glycerine, polyunsaturated fatty acids used to produce pharmaceuticals, flavours, fragrances and cosmetics
Alcohol	α-Amylases	Liquefaction of starch or fragmentation of gelatinized starch
	Amiloglucosidase	Saccharification or complete degradation of starch and dextrins into glucose
Starch and sugar	α-Amylases	Enzymatic conversion of starch to fructose: liquefaction, saccharification and isomerization
		Liquefaction of starch
	Glucoamylase and pullulanase	Saccharification
	Glucose isomerase	Isomerization of glucose
Animal feed	β-Glucanases	The reduction of β-glucans
Brewing industry	β-Glucanases	The reduction of β-glucans and pentosans
Fine chemical	Lipases, amidases and nitrilases	Enantiomeric intermediates for drugs and agrochemicals
		Hydrolysis of esters, amides, nitriles or esterification reactions
Leather	Lipases	To remove fats in the de-greasing process
Textiles	Amylases and cellulases	To produce fibres from less-valuable raw materials
Pulp and paper	Xylanases	Used as a bleaching catalyst during pre-treatment for the manufacture of bleached pulp for paper

(Giorno et.al, Biocatalytic membrane reactors: applications and perspectives, TIBTECH AUGUST 2000 (Vol. 18),339-349)

2.3 Applications of biocatalysts in MBR

The enzymatic bioconversion processes are of increasing use in the production, transformation and valorisation of raw materials. Important applications have been developed in the fields of food industries, fine chemicals

synthesis (particularly in the pharmaceutical area) or even for environmental purposes.

2.3.1 Membrane bioreactor in Sugar and Starch processing

Sugar and starch industry represent the competitive world sugar market, Table 2.2 summarizes major sugar productive countries and their production. Application of membrane technology in sugar industry contributes to the sustainable development in the field. In particular, clarification of sugarcane juice, production of glucose or glycerol as well as are sugar related products are aspects where membrane bioreactor can play a role.

Table 2.2: Major sugar producing countries

2007/08 est.	PRODUCTION MILLION TONS	EXPORTS MILLION TONS	PER CAPITA CONSUMPTION KGS
BRAZIL	31.355	20.957 [1]	58
INDIA	28.804	3.298 [4]	20
EU	17.567	1.400 [8]	34
CHINA	14.674	-	11
THAILAND	8.033	5.288 [2]	36
UNITED STATES	7.701	-	29
MEXICO	5.978	0.350 [15]	52
SADC	5.834	2.410 [5]	22
AUSTRALIA	5.013	3.750 [3]	47
PAKISTAN	4.891	-	25

In the confectionery and many food and beverage industries, sugar (present as starch, sucrose, fructose and glucose, etc.) is the main constituent in some of the

process streams. Inevitably, it is also present in the effluent streams arising from these industries. There is, however, considerable interest among manufacturers to optimise process economics through product recovery, and to respond to environmental pressure to reduce the waste generation.

Enzymatic hydrolysis of starch is traditionally performed in large volume batch reactors using soluble enzyme following a two-step procedure including the liquefaction and saccharification [Kędziora et al 2006]. But nowadays it can be performed in a single steps by using amylase enzyme termamyl which is able to produce dextrins [Paolucci-Jeanjean et al 2000a, b] using membrane reactor.

First of all, the starch being dissolved in water and partially hydrolysed with an α-amylase to give maltodextrines and in the next step, saccharification enzymes transform liquefied starch into low molecular weight oligosaccharides such as glucose or maltose. The conventional batch reaction processes has a great number of disadvantages such as incompatibility of enzyme recovery and reuse, high labour and purification cost, high capital investment and discrepancies in glucose syrup quality, low efficiency, batch to batch variations, and most of all, the high enzyme cost. But the application of membrane reactors make it possible to continuous operation in lower reactor volume as well as in shorter reaction time [Paolucci-Jeanjen et al 2000 a, b][Sims et al 1992 a, b][Słomińska et al 1998] increase the reactor's efficiency and finally reuse enzymes in a continuous way.

The application of membrane reactors in starch industry is particularly used in the production of smaller assimilable sugars. This reaction is carried out in system in which the enzyme is not immobilized and the membrane works as separation

device (MBR). The enzymes used are amylolytic enzymes and debranching enzymes [Gaouar 1997] or using liquefied starch as substrate (**Table 2.3**)[Grzeskowiak-Przywecka A.2007]. The major problems in the application of the membrane reactor are large decrease in permeate flux due to concentration polarization and fouling [Paolucci-Jeanjen D., 2000, a, b][Paolucci-Jeanjen D., 1999]. Different solutions are applied to decrease fouling phenomena such as the pre-treatment of the raw starch solution. Other studies were devoted to the examination of factors that mainly affect membrane performance such as: molecular-weight cut-off, enzyme dosage, residence time, transmembrane pressure, carbohydrate composition, and retention factor [Grzeskowiak-Przywecka, 2007].

Membrane bioreactors have been used for production of glucose, maltose, maltotetraose, and cyclodextrins [Paolucci-Jeanjen et al 2000 a, b][Sims et al 1992 a, b][Słomińska et al 1998][Słomińska 2002 a, b][Lopez-Ulibarri et al 1997][Kędziora et al, 2001] for the food grade industrial production [Paolucci-Jeanjean et al 1999] like puddings, jellies, and fruit desserts. In this system hydrolysis can be carried out simultaneously by separating syrups from enzymes and non-hydrolysed starch [Paolucci-Jeanjean et al 1999]. An extra separator system to extract the product is not necessary but it is needed to concentrate the product by application of different advanced membrane operations (UF/NF).

Table: 2.3 Examples on the use of membrane Bioreactors for sugar production

Starch used	Enzyme Used	Membrane used	Molecular weight cut off(kDa)	Reactor type	Reference
Commercial potato starch	α-amylase (BAN 480L)	Tubuler ceramic	50	MBR	[Hoq et al.1985]
Cassava	Termamyl	Carbosep M4	50	BMR	Malcata et.al, 1992]
Cassava	maltogenase and Promozyme	Carbosep M4	50	BMR	[Goto M..1922]
Amylos	amylolitic enzyme complex—from fermentation of whole wheat flour by Aspergillus awamori	hydrophilic cellulose acetate	40	MBR/BMR	[Rucka et.al,1990[

Another field of membrane bioreactor application is the production of cyclodextrins or oligosaccarides. The development in this field was pushed from the high interest devoted to this compound in the last period, due to the fact that they have applications in several fields, including food pharmaceutical, cosmetic, plastic industry as emulsifiers, antioxidants and stabilizing agents. The production of cyclodextrins by membrane bioreactors was conducted using different starting source includig corn starch and soluble potato. A recent work reported their production also starting from tapioca starch [Sakinaha 2007].

The production of oligosaccarides to be used as functional food was also obtained by the immobilization of dextranase on polymeric matrix [Torras 2008].Cyclodextrins can be used as carriers for molecular encapsulation of flavours and other sensitive ingredients [Lajos Szente 2004]. The molecular encapsulation of

lypophilic food ingredients with cyclodextrin improves the stability of flavours, vitamins, colorants and unsaturated fats, etc.

Various types of oligosaccharides have been found as natural components in many common foods including fruits, vegetables, milk, honey. Oligosaccharides can also be used as functional food ingredients that have a great potential to improve the quality of many foods. In addition to providing useful modifications to physicochemical properties of foods -such as the improvement of intestinal microflora based on the selective proliferation of bifidobacteria, stimulation of mineral absorption, non- or anticariogenicity, and the improvement of both plasma cholesterol and blood glucose level.

Basically oligosaccharides are short-chain sugars generally consisting of two to ten building block small sugars. It is using as a nutrition and food ingredients and additives. Apart from directly extraction from plant the oligosaccharides can be processed by enzymatic synthesis using enzymes which possess hydrolytic or transglycosylation activity, in continuous membrane bioreactor. Both batch reactor with soluble enzymes and continuous system with enzymes or whole cells immobilized have been used.

2.3.2 Membrane bioreactor in Oil & Fat processing industry

The use of membrane bioreactor for the hydrolysis of oils and fats is intensively investigated. The biocatalysts used are mainly lipases and esterases and the process in which they are involved for functional food production are

esters synthesis to produce emulsifiers and aroma compounds and oil hydrolysis for free fatty acids, mono or diglycerides productions.

Monoglycerides, diglycerides, triglycerides, glycerol are widely used in the food industry as emulsifiers for bakery products, margarines, dairy products, confectionery, etc. In foods and beverages, glycerol serves as a humectant, solvent and sweetener, and may help preserve foods. It is also used as filler in commercially prepared low-fat foods (e.g. cookies), and as a thickening agent in liqueurs. Although it has about the same food energy as table sugar. The glycerin has many uses, such as in the manufacture of food and in the production of pharmaceuticals too. The most commonly used products are glycerol monostearate, monooleate, and monoricinoleate [Meffert 1984].

The complex mixtures that contain 40 –48% monoglycerides (MG), 30–40% diglycerides (DG), 5–10% triglycerides (TG), 0.2–9% free fatty acids (FFA), and 4–8% free glycerol are generally termed monoglycerides. These mixtures have applications in food fats (margarine, ice cream, sweets, etc). Pure monoglycerides (90–97%), obtained by molecular distillation of the above mixtures, are also commercially available. The higher-purity monoglycerides are preferred for bakery uses because of their good amylase complexing ability. Most commercial MG are produced from edible, refined, hydrogenated animal fats (tallow, lard, etc.) or from hydrogenated vegetable oils (palm, soybean, corn, olive, peanut etc.). High oleic vegetable oils can also be used as raw materials for the production of emulsifiers for liquid and low-fat margarines.

The monoglycerides can be produced on an industrial scale by glycerolysis of fats and oils by means of inorganic alkaline catalysts, such as sodium hydroxide or by enzymatic route. Application of enzymes as catalysts for reactions in the oils and fats industry is being extensively studied in the litrature. Enzymes are chosen since they show many advantages over traditional inorganic catalysts: they have large catalytic activity under mild operating conditions; they show large selectivity to the desired product with no significant side reactions, leading to products of high purity.

Various review papers about membrane bioreactors using lipase in vegetable oil and fat processing have been published during the last decades [Balcão 1996] [Giorno 2000] [Rios 2007].

The influence of operating conditions of lipase immobilized in a two-separate phase membrane bioreactor has been reported [Giorno, 1997, 1998, 2003]. In particular, the effect of immobilization method, amount of enzyme, hydrodynamic conditions, and microenvironment conditions (such as pH, temperature, membrane material) have been investigated [Li 2003]. Strategies to improve reaction performance as well as transport properties through the enzyme-loaded multiphase system have been exploited [Giorno 2006, 2007]. Three different enzyme membrane reactors have been compared as illustrated in the **Fig. 2.4**. Lipase was used as free in a stirred tank reactor, and as immobilized in membrane in absence and in presence of oil/water droplets. The use of oil droplets immobilized together with the enzyme significantly improved the performance of the system thanks to the positive effect of the o/w interface uniformly distributed

through the membrane on the enzyme activity as well as on the substrate transport.

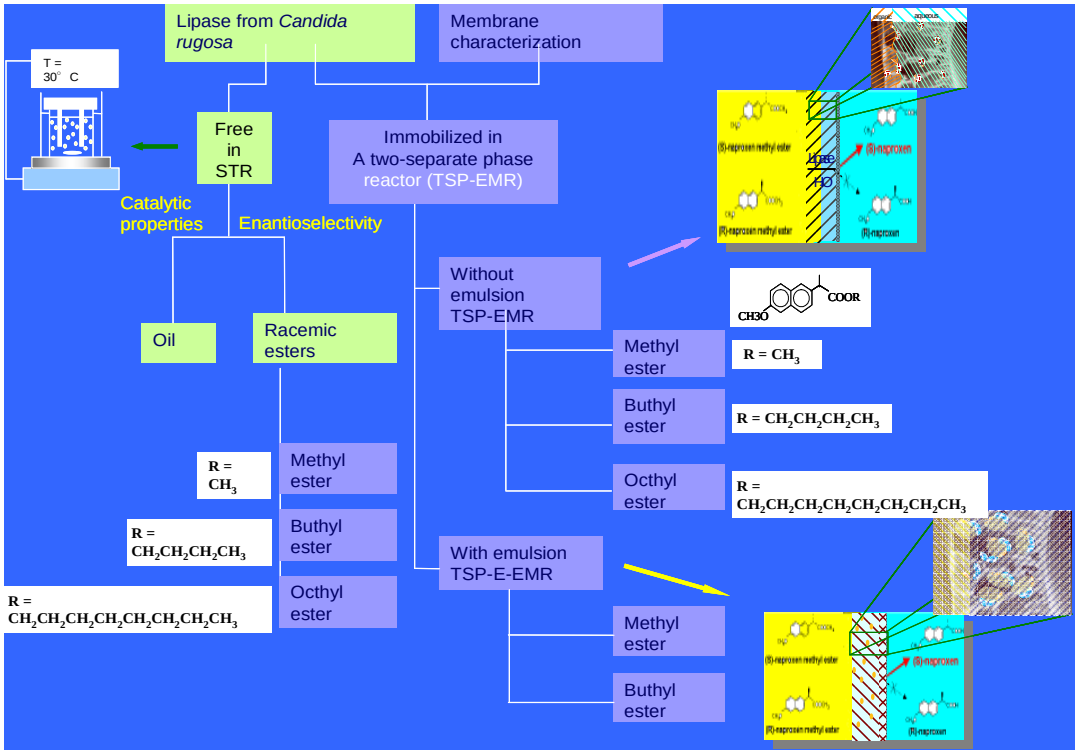

Fig. 2.4. Schematic representation of bioreactors studied (Giorno 1997, 1998, 2003) using lipase as biocatalyst: free stirred tank reactor, two-separate phase enzyme membrane reactor and two-separate phase enzyme membrane reactor with emulsions.

Lipase has been immobilized on polymer membranes with hydrophilic [Bélafi-Bakó 1994] [Knezevic 2004] hydrophobic [Mohammad Mozammel Hoq et al 1984] properties, as well as on inorganic membranes [Mameri 2000]. Another application of membrane bioreactor is production of specific structured lipids in enzymatic route from rapeseed oil and capric acid [Xu 2000]. Production of ω3-polyunsaturated fatty acid (ω3-PUFA) concentrates from fish liver oils (which have been claimed to provide beneficial health effects via prevention of coronary

heart diseases) for use as nutro-pharmaceutical food supplements is another application of lipase in membrane bioreactor, and sequential lipase- catalyzed chemical incorporation in triglycerides. Lipase from *Candida rugosa* was also immobilized on Cuprophane membrane [Knezevic et al 2004] in a hollow-fiber module.

2.3.3 Membrane Bioreactors in Hard drink industry and liquid beverages

Wine

Membrane bioreactors are developing in wine field for the production of aromatic compounds and flavour by the use of glucosidases, the production of additives from pectinase hydrolysis, and the production of preservatives molecule such as lactic acid by the use of malolactic bacteria.

The production of wine in terms of cropped surfaces and product yield fluctuates in a significant way during the years. At the end of 90's the production has the tendency to decrease, but a significant increase was achieved at the end of 2004/2005 going back toward another decrease in 2005/2006, where the production was a 4% less. Nevertheless the production of European countries (27 countries) in 2007 was about 174449.170 (Wine, production - 1 000 hl). Respect to the total worldwide production, Europe represents the higher producer of wine (http://news.reseau-concept.net/images/oiv/client/STATISTIQUE__Verone_2008_EN_definitif_41diapos.pps#1) having leading countries like France, Italy and Spain. The United States is another important producer followed by Argentina and China, while the economy of other countries like Germany, South Africa and Chile, is growing in the last three years.

Thanks to the action of different yeasts, both *Saccaromyces* and non-*Saccaromyces* type, in the first part of wine making there is the conversion of glucose in ethanol CO_2 and other products. The presence of the yeast is fundamental in must fermentation due to the production of particular enzymes that helps the fermentation process. The use of these type of enzymes or directly the yeasts with this enzymatic activity, immobilized with membrane or on other support for wine fermentation is of high interest.Some examples of coupling the enzymes useful in wine making and membrane reactors are reported in Table 4.β-glucosidase is an important enzyme in wine making, the enzyme is employed in different application like production of rosé wine from red grapes, for the hydrolysis of antocianines, and for the hydrolysis of terpenglucosides etc. [Martino 1996]. The immobilization of this enzyme, or bacteria and yeast showing that enzymatic activity is of high interest in beverages production with enhanced aroma. In literature are reported some examples [Gallifuoco 1999] [Martino 1996] about the immobilization of β-glucosidase on different support (Cellulose PEI (Baker), alpha-aluminia CT 2000 (Alcoa Chemie), gamma-aluminia (Akzo), chitosan (Chitobios) and polymeric) applying adsorption, covalent bonding by glutaraldeide and cross-linking immobilization techniques.

Some immobilized glucosidase enzymes has been also proved on pilot scale. They were used on a continuous flow stirred tank membrane reactor in a model system and also during wine-making [Gallifuoco 1998 Gallifuoco 1999]. In this system the enzyme was immobilized on chitosan pellets and to simulate the natural process, the medium was also supplemented with chemicals present in

the wines (fructose, ethanol, nerol, linanol, geraniol). Fructose did not decrease biocatalyst stability, while alcohol affected enzyme half-life from 2586 h at 3% (w:v) ethanol to 1378 h at 12% (w:v).

Enzyme stability was not dependent on substrate concentration and was considered satisfactory for an industrial process (half-life 1.2 years). Many precursors of the aromatic components of wine are monoterpenes (geraniol, nerol, citronellol, linalool, a-terpineol,etc.) in di-glycosidic form, that contain b-D-glucopyranose bound directly to aglycon and/or other sugars among which are a-L-rhamnopyranose and a-L-arabinofuranose. Therefore, to develop the aromatic potential of a wine to the full, together to ramnopyranose (Rha), it is also necessary to utilize the other glycosidases: a-L-arabinofuranosidase (Ara, EC 3.2.1.55), and first of all b-D-glucopyranosidase (bG, EC 3.2.1.21). and a-L-rhamnopyranosidase (Rha, EC 3.2.1.40).

An important reaction that occurs in wines and in particular in white wine and in rosé is the development of madeirized flavour and the tendence to browing. These processes are mainly due to polyphenols, that can have also beneficial health effect because of their antioxidant properties. Oxidative enzymes like laccase coming from fungi are used to improve the process. Several studies were performed on the use of Laccase in phenol removal processes for must and wine stabilization [Duran 2002]. Laccase was immobilized on different membrane material applying different immobilization techniques from different sources (**Table 2.4**).

Cantarelli and Giovanelli (1991] carried out assays in order to determine if the enzymatic preparations could be used in white wines production for polyphenols reduction in musts (and consequent stabilization of the wine colour) instead of oxidation. The results demonstrated that the enzymatic treatment coupled with filtration with polyvinylpolypyrrolodone (PVPP) reduced the quantity of oxidized polyphenols.

Table 2.4 Examples of laccase immobilization on different membrane material

Biocatalyst	Membrane mater	Immobilization	Reference
Laccase from *Aspergillus sp.*	Nylon 66	Adsorption	Zamorani Duran 2002
Laccase from *Pyricularia oryzae*	Polyethersulphone membranes	Adsorption	Lante 2000
Laccase from *Trametes versicolor*	Hydrophilic PVDF microfiltration membrane	Covalent binding	Jolivalt 2000
Laccase from *Trametes versicolor*	polyether sulph one membranes	Entrapment	Júnior2008

Other important enzymatic activities in wine making and in particular in wine clarification process are pectinases, this are usually used to improve processability and to produce additives. The main membrane bioreactor configuration used in wine industry using pectinases action is free enzyme membrane reactor (BMR). The soluble enzyme is confined in the retentate side of the membranes where it is in contact with the substrate. In the **Table 2.5** some examples and membrane material used for the pectines hydrolysis are reported. These applications are referred both to the wine and fruit juice treatment.

Table 2.5 Examples and membrane material used for the pectines hydrolysis

Biocatalyst	**Membrane**	**Bioreactor configuration**	**Application**	**Reference**
pectin lyase from *Penicillum italicum*	Ultrafiltration membrane	MBR	Production of pectic oligosaccarides	Alkorta 1995
polygalacturonase from *A. niger*	30 kDa flat regenerated cellulose membrane	MBR	Production of D-galacturonic acid	Bélafi-Bakò 2007
Polygalacturonase and pectin lyase from *A. niger*	Spiral wound polysulfone membrane (10 kDa)	MBR	Wine clarification	Rodrigue-Nogales 2008
Endo-polygalacturonase from *Aspergillus pulverulentus*	Amicon 10 kDa	MBR	Production of pectic oligosaccarides	Olano-Martin 2001
Polygalacturonase from *A. niger*	Titania microfiltration	BMR	Production of pectic oligosaccarides	Szaniawski 1996
Rapidase liquid plus	Polyvinilidene fluoride tubular, polysulphone spiral wound	BMR	Apple juice clarification	Giorno 1998 a,b
Amylase and pectinase	Polysulfone single-hollow fiber	BMR	Fruit juice processing	Carrìn 2001
Commerical pectinaase	Hollow fiber ultrafiltration	BMR	Fruit juice processing	Carrìn 2000
Endopectidase from *A. niger*	10 kDa Spiral wound polysulfone	MBR	Apple pectin hydrolysis	Rodriguez-Nogales 2008.

Together with protein immobilization, the alternative strategy for wine-making, is cell immobilization. Although this application is rapidly expanding in research area, the development at industrial scale is still limited. Takaya et al. [2002) studied the efficiency of two membrane bioreactor systems for continuous dry wine-making. The first configuration was a single-vessel bioreactor, while the second configuration included two vessels; one operated as a continuous stirred tank reactor and the other was a membrane bioreactor. The double vessel resulted 28 times more productive that the single-one.

Cell immobilization is rapidly expanding research area. The purpose to use this technique is to improve alcohol production and overall product aroma, taste and quality. Many support are used for cell immobilization in this field divided in inorganic, organic and natural material. Some examples of different supports and its main application is reported in **Table 2.6**.

Table 2.6 Materials used for cell immobilization

Inorganic material	Immobilized cell	Application	Reference
Mineral kissis	*Saccaromices*	Aroma improvement	Bakoyianis 1992
γ- aluminia	*Saccaromices*	Aroma improvement	Loukatos 2000
Organic support	Immobilized cell	Application	Reference
Cellulose covered with Ca-alginate	*Saccaromices and Candida*	Enhance glycerol formation in wine	Otsuka 1980, Ciani 1996
Ca-alginate beds	*Saccaromices*	Must fermentation	Suzzi 1996
Natural support	Immobilized cell	Application	Reference
Delignified cellulose Gluten pellets	*Saccaromices*	Fermentation Production of wine with less alchol content	Bardi 1994, 1996 1997

Malolactic fermentation is a secondary process that occurs in wines during the maturation period. Lactic bacteria predominately of the genera *Oenococcus, Lactobacillus* and *Pediococcus* are responsible of this process, where L-malic acid is converted to lactic acid, important food preservative, and carbon dioxide. As a consequence of this reaction the total acidity of the wine decreases. *Oenococcus oeni* can carry out this process in one step, without the production of piruvic acid. Other by-product produced during this fermentation can affect wine flavour. Also some yeast as *Saccaromyces* can convert malic acid through maloethanolic fermentation [Redzepovic 2003].

The immobilization technology is important also in this field, where the cell compartimentalization can help to i) increase the tolerance towards malolactic fermentation bacteria, ii) to develop the desired flavour selecting the appropriate cultures, iii) acceleration of the process increasing cell densities, iv) re-use of the cell.

A kinetic analysis was carried out using three different immobilization techniques of malic enzyme for the development of a membrane bioreactor: 1) polymeric membranes [Giorno 2001] and cross-linking reaction, 2) within polyurethane foams, and within a gel like membrane formed on active side of ultrafiltration polymeric membranes [Iorio 1985]. Enzymatic cell-free reactors, did not allow to efficiently achieve complete and rapid consumption of the L-malic acid [Formisyn 1997][Maicas 2001].

2.3.4. Beer

Beer is the second most consumed beverage in the world behind tea, and it continues to be a popular drink. The brewing industry has an ancient tradition and is still a dynamic sector open to modern technology and scientific progress. Brewers are very concerned that the finishing techniques they use are the best in terms of product quality and cost effectiveness [Fillaudeau 2001].

Beer production requires about seven days of fermentation and large scale fermentation and storage capacity. The main field in which membrane bioreactors can be developed in beer are the alchohol free beer and in the maturation and aroma control.

In the first process the two main approaches currently used are the removal of the alcohol from product and limited fermentation. In the case of limited fermentation the system is most efficient where the fermentation-cell are immobilized. The yeasts commonly used for this process are *S.cerevisiae.* Different kind of support are used to immobilize the yeasts in brewing, they can be divided in inorganic, organic and natural. The prevalent organic support are: Polyethylene, PVC, Polysaccarides, DEAE-cellulose; the inorganic porous ceramic and silicon and the natural support are Delignified cellulose and Gluten pellets [Kourkoutas 2004].

2.3.5 Ethanol production

The requirement of ethanol in beverage industries as additive use has been steadily increasing and so is the pursuit of immobilized microbial cell systems for ethanol fermentation. Research on alcohol production usually focuses on volatile by-product formation, because these constituents are critical parameters for distillates and alcoholic beverages quality. For ethanol production different yeast strains are used as: *S. cerevisiae, S. diastaticus, K. marxianus and Candida sp.,* and different bacteria like *Zymomonas mobilis.* The requirement for food grade purity is not essential due to the employment of a distillation step. A membrane distillation bioreactor was developed for ethanol production [Gryta 2000, 2001], where the batch fermentation was coupled with a membrane distillation process. The porous capillary polypropylene membranes were used for the separation of volatile compounds from the feed . The elimination of these compounds allows

increase in ethanol productivity and rate. In this case the yeast used was *S. cerevisiae.*A membrane bioreactor for the production of ethanol was developed in a pilot plant [Escobar 2001]. This system integrated ceramic microfiltration membranes with a stirred-tank bioreactor.

2.4 Membrane bioreactor application in liquid beverages

The main applications of membrane bioreactors in other drink industry are: reducing the viscosity of juices by hydrolysing pectins, reducing the lactose content in milk and whey by its conversion into digestible sugar.

2.4.1 Fruit juices production

The production of fruit juices is divided in six major steps: crushing, pressing, clarification, centrifugation or filtration, concentration, pasteurization. During the fruit crushing there is the solubilization of pectins, these compounds usually can affect the processability creating turbidity and cloud forming. Pectinase, the pectolityc enzyme responsible for pectins hydrolysis are commonly used in fruit juice industry, in two steps: pressing and clarifications.

During pectins hydrolysis it is also produced the monomer of pectin, $_{D}$-galacturonic acid, which is an important compound, raw material in the food, pharmaceutical and cosmetic industry to manufacture e.g vitamic C, or acidifying, tensioactive agent.

Oligosaccarides derived from pectins hydrolysis can also have some important applications as repressors of liver lipid accumulation in rats [Yamaguchi 1994], as antifungal phytoalexin-elicitors in plants [Bishop 1984], inducers of flowering and antibacterial agents [Isawaki 2001].

Traditionally, enzymatic hydrolysis of pectins has been conducted in batch systems. Unfortunately, after each cycle of operation the enzyme could not be recovered for further use and immobilized enzyme could suffer from steric hindrance effects and losses in enzyme activity as a result of immobilization . The use of membrane bioreactor is the alternative efficient strategy, in which the enzyme is retained or compartimentalized, thus increasing enzyme utilization. One of the membrane bioreactor configuration commonly used is with the enzyme compartmentalized in the retentate side of the membrane together with the substrate, while the product is separated in the permeate.

Different works were carried out for pectins hydrolysis in membrane bioreactor systems using free enzyme membrane reactor. Alkorta et al. [1995] studied the reduction in viscosity of pectins catalyzed from pectin lyase from *Penicillum italicum* in a membrane reactor. This enzyme results as the only pectinase enzyme capable of hydrolysing α-1,4 glycosidic bond of highly esterified pectins, without altering the volatile compounds responsible of aroma of various fruits [Alana 1990, Alkorta 1995] the reduction in viscosity was demonstrated with high efficiency towards different fruit juice: grape, peach, melon, apple and pear, showing a little decrease in the case of apple and pear juice.

Another biocatalyst used frequently in pectin hydrolysis was polygalactorunase from *A. niger*. A. niger pectinases are most widely used in industry because this strain posses GRAS [Generally Regarded as Safe) status, so the metabolites coming from its production can be directly used without further treatment [Gummadi 2003]. The pectinases produced from this strain are: polymethylgalacturonase (PMG), polygalacturonase (PG) and pectinesterase. However, particular pectinases are used for specific purpose, for example only polygalacturonnase is used for baby food products [Gummadi 2003].

A recent work reports the use of polygalactorunase from *A. niger* in flat sheet membrane reactor, which shows excellent stability for more than 50 hours. In this case the membrane used was 30 kDa regenerated cellulose membrane [Belafi-Bako, 2007]. The same biocatalyst was used in a free enzyme membrane reactor where the membrane used was a spiral wound polysulphone membrane (10 kDa MWCO), attaining a conversion of 83 % and a stability for a long term period (15 day) [Rodriguez-Nagales 2008]. The performance of pectins hydrolysis was also tested immobilizing directly the enzyme on the membrane and conducting the reaction in a biocatalytic membrane reactor [Alkorta 1995]. The use of pectinases immobilized on ultrafiltration membrane hydrolyze the pectin to lower molecular weight species permitting an extension of membrane operation without cleaning [Carrin 2000].

Pectinase was also immobilized by physical immobilization on titania microfiltration membrane [Szaniawski 1996] and on polysulphone hollow fiber membrane [Carrin 2000], and co-immobilized with amylase on polymeric hollow

fibre membrane to hydrolyse simultaneously starch and pectins. The co-immobilization showed an improvement of flux of 35% [Carrin 2001].An integrated membrane process for producing apple juice and apple juice aroma concentrates was proposed by Alvarez et al [2000]. The efficient system involves the following operations: an integrated membrane reactor to clarify the raw juice; reverse osmosis to pre-concentrate the juice, pervaporation to recover and concentrate the aroma compounds, and final an evaporation step to concentrate apple juice. These operations were tested in laboratory and pilot plant units, giving promising results both on the yield of product and also for economical aspects. Some examples of immobilized pectic enzyme are present at industrial scale [Alkorta 1998] [Giorno 1998]

2.5 Functional food production in milk and whey field by membrane bioreactor

The first application on a large scale of a membrane bioreactor was the hydrolysis of lactose by immobilized β-galactosidase on cellulose fiber for the production of milk with law lactose content [Pastore 1976]. Lactose, together with high molecular weight proteins are allergenic compounds present both in milk and whey. Intolerance to milk borns from the fact that some subject can not digest proteins, contained in milk and whey, with a molecular weight higher than 5 kDa.

2.5.1 Lactose hydrolysis

Lactose is the dominant carbohydrate in milks and it is also contained in whey. A large number of people do not digest lactose properly due to the lack or inactivity of the intestinal β-galactosidase and they suffer from intestinal dysfunction. Besides of this, lactose is a sugar with high BOD, low sweetness, and low solubility and has a strong tendency to adsorb flavours and odours compared to its hydrolysis products; glucose and galactose. Lactose hydrolysis is an important food process, not only to produce free lactose milk, but also to improve processes for the production of refrigerated diary products, because some technological difficulties occurs associated with lactose crystallization [Sener 2006]. Another important application of lactose hydrolysis is the production of additives, like lactic acid, glucose and galactose that can be used in human diet [Ladero 2000]. For the industrial applications of enzymes to the productions of large quantities of product, the enzymes should be immobilized to be used in continuous reactors. Several procedures for β-galactosidase have been studied: entrapment, adsorption, ionic interaction, affinity, complex formation with metal, and covalent bonds [Ladero 2000].

Several reactors were also tested using different membrane reactors configuration and different starting sources. In the **Table 2.7** some examples showing support material application are reported. The main enzyme used in membrane bioreactor for lactose hydrolysis are from *Kluyveromyces* yeast and *Aspergillus* fungi, microorganisms considered safe (GRAS). In particular the enzymes from fungi can be used in acid wheys since their optimum pH is 3.5-4.5,

while the enzymes from yeasts can be used in milk and sweet wheys since their optimum pH is between 6.5-7 [Santos 1998]

Table 2.7 Examples of Membrane bioreactor used to hydrolyse lactose

Biocatalyst	Source	Material and reactor configuration	Application	Reference
B. circulans	skimmed milk	MBR	High quality milk	Bakken 1989
K. lactis, A. oryzae	lactose	MBR with ceramic membrane	Production of galactosil-oligosaccarides	Czermak 2004
β -glycosidases from the archaea *Sulfolobus solfataricus* (Ss β Gly) and *Pyrococcus furiosus* (CelB)	Lactose	MBR with an ultrafiltration unit	Production of oligosaccarides	Petzelbauer 1999
β -galactosidase from *Kluyveromyces lactis*	Lactose	BMR	Galactose and glucose production	Neuhaus 2006
β-galactosidase commercial enzyme	Lactose	MBR	Production of oligosaccarides	Jurado 2006
A. orza, K. lactis	Lactose	MBR	Production of Galactosyl-oligosaccharides	Gonzalez 2009

As previously mentioned the other application of membrane bioreactor in the lactose hydrolysis is the production of lactic acid. Lactic acid is one of value-added product produced from processing cheese whey. The food and drug administration have approved lactic acid and its salts to be GRAS [Harington 2006]. The bacteria usually used for the production of lactic acid by fermentation process are from cheese whey are *Lactobacillus helveticus* **[**Tango 2002], [Roy 1986], [Li 2006] and *Lactobacillus casei*, while *Bifidobacterium longum* converts

lactose into lactic acid and produce antibacterial compounds [Gomes 1999]. The main configuration of membrane bioreactor for the production of lactic acid is a fermentation reactor with a membrane unit as reported in Table 2.8 . In this kind of configuration cells, protein and lactose are separated by a filtration unit and returned to the fermentor while lactic acid is separated in the permeate. Some examples of biocatalytic membrane reactors are also present in literature. *L helveticus* cell were immobilized in polymeric membrane reaching a lactose conversion of 79 % and lactic acid yieald of 0.84 g of lactic acid /g of lactose utilized [Sahbazi 2005].A two stage continuous fermentation with membrane recycle has been studied that enhance lactic acid productivity from 21.6 g/dm^{3}h in a single stage to 57 g/dm^{3}h in two stages [Kwon 2001].

Table 2.8. Examples of Membrane Bioreactor in the production of lactic acid

Biocatalyst	Source	membrane reactor configuration	Reference
L. ramnosus	glucose	MBR	Choundhry 2006
L helveticus	whey	MBR	Shahbazi A 2001
L. casei	lactose	MBR	Olmos-Dichara 1997
L. ramnosus	lactose	MBR	Shahbazi 2001

2.5.2 Protein hydrolysis in milk and whey by MBR

The hydrolysis of high molecular weight proteins into small polipetides is an alternative approach to produce low allergenic (β-lactoglobulin) fresh milk. The possibility to hydrolyse high molecular weight proteins by membrane bioreactors provides rich source of peptides which are latent until released and activated, e.g. during gastrointestinal digestion or milk fermentation. Once activated, these peptides are potential modulators of many regulatory process.

Milk derived bioactive peptides can have physiological functionality on cardiovascular, (antihypertensive, antioxidative, antithrombotic, hypocholesterolemic), nervous (agonistic, anthagonistic oppioid activity), gastrointestinal (anti-appetizing, antimicrobial) and immune (antimicrobial, immunomodulatory, citomodulatory effect) system [Korhonen 2006]. The active peptides can be produced by the hydrolysis of digestive enzymes, through proteolytic microorganism and through the action of proteolytic enzymes derived from microorganisms or plant. Some examples are reported in **Table 2.9** .

Table 2.9 : Examples of Biocatalyst used to produce active peptides

Biocatalyst	Protein source	Active peptides produced	Reference
Pepsin	Casein	(ACE)inhibitory peptides	Korhonen 2006
Trypsin	Casein	(ACE)inhibitory Calium binding phosphopeptides	Fitz-Gerald 2004 Gobbetti 2004
Protease N	Whey protein	different peptides	Cheison 2005
Lactococcus lactis	Casein, milk	(ACE)inhibitory peptides	Korhonen 2006
Lactococcus helveticus	Casein, whey proteins	(ACE)inhibitory peptides	Nakamura 1995
Lactobacillus delbruecki ssp. vulgaris	Casein	(ACE)inhibitory peptides	Gobbetti 2000

Commercial production of bioactive compounds from milk proteins is limited. The use of enzymatic membrane reactors for continuous production of specified peptide sequences was introduced during 1990. Nowadays it has been widely studied, in literature, for total conversion of food proteins of various origins with improved nutritional and/or functional properties. Continuous extraction of

bioactive peptides in membrane reactors has been mainly applied to milk proteins using different membrane material and different membrane reactor configuration (See **Table 2.10**).

Table 2.10 Examples of production of bioactive peptides using MBR

Biocatalyst	Substrate	Membrane reactor configuration	Application	Reference
Alcalase	casein	MBR	production of peptides	Mannheim (1990)
Trypsin	caseinomacropeptides	MBR with ultrafiltration unit	recovery of antithrombotic peptides	Bouhallab 1995
Pepsin	goat whey	MBR	production of α-lactorphin	Bordenave 1999
Trypsin	milk protein	BMR using polyacrilamide membranes	production of phosphopeptides	Righetti 1997
Trypsin Chymotrypsin	whey protein concentrate (WPC) and heat treated WPC	MBR with ultrafiltration unit	production of polypeptides and rich fraction of small peptides	Turgeon et al 1990
Pepsin, Trypsin, Chymotrypsin, Pancreatin, Elastase, Carboxypeptidase	α-lactalbumin and β-lactoglobulin	MBR with two step ultrafiltration system (30 and 1 kDa)	production of ACE-inhibitory peptides	Pihlanto-Leppala 2000
Protex 6 L from *bacillus licheniformis*	whey protein	MBR	production of whey protein hydrolisates	Prieto 2008
Hydrolytic enzymes	whey protein hydrolisates	MBR with ultrafiltration unit	production of emulsifyng peptides	Gauthier 1996

During protein hydrolysis by membrane bioreactor it has to be considered that an excessive hydrolysis should be avoided because high content in free aminoacids involves negative effects like bad sensory properties bad high osmolarity [Guadix 2006]. This means that to develop the system at industrial scale, the hydrolytic reaction has to be strictly controlled. Different works were focused on the optimization of process parameters for a continuous production of

whey protein hydrolysates. Guadix et al [2006] developed a MBR with an ultrafiltration unit (Polyethersulphone) where no effects on enzyme activity, due to mechanical shear stress, adsorption to the membrane or enzyme leakage were observed. The effect of temperature on the performance of a batch reactor with a ultrafiltration unit made of polysulphone material of 8kDa was analysed in the hydrolysis of a whey protein hydrolisates [Prieto 2008]. The experimental data perfectly fit a mechanistic model also proposed in the same article.

2.6 The production of pharmaceuticals using biocatalytic membrane reactors

The different systems that have been used for the production of amino acids, antibiotics, anti-inflammatories, anticancer drugs, vitamins, and so on, are summarized in Table 2.11. Many studies have also focused on the production of amino acids, arylpropionic acids, amines and carboxylic acids (Giorno. et al.1998, Goto 1996, Yang 1997, Battistel 1991, Timmer1 994, Moueddeb 1996). The major problems in the production of these compounds on a large scale include: (1) the requirement for expensive cofactors; (2) the low water-solubility of the substrates; and (3) the separation and purification of the products from complex solutions. However, studies carried out mainly on a laboratory scale indicate that in many cases the use of the appropriate type of membrane and membranereactor design can overcome these difficulties. In reactor systems using coenzyme-dependent reactions, negatively charged membranes are used to retain the cofactor in the reaction vessel. Retention is obtained by electrostatic repulsion

between the negatively charged cofactor and the membrane. heterogeneous reaction is carried out in an emulsioned organic–aqueous system where the organic phase contains the substrate, the product is extracted in the aqueous phase and the enzyme is adsorbed at the interface. The use of biphasic membrane systems in side stream configuration that contain the enzyme and keep the two phases separated (Hallstrom 1990, Lopez 1997) (but in contact with the membrane) can solve most of the problems presented by the traditional systems. The most frequently used enzymes are hydrolases, in particular, lipases. Enzymes in organic media are able to work in microenvironments that contain very little quantities of water (usually less than the solubility limit) and several studies have confirmed that it is possible to carry out biotransformations in organic media (Tsai 1993, Klibanov 1985). Immobilized enzymes operating in organic media show novel properties such as enhanced stability and altered substrate specificity. Several studies carried out using lipases immobilized on polymeric membranes have demonstrated these effects. The use of a hydrophilic matrix can help to protect the biocatalyst because it helps to maintain the water molecules around the enzyme, and inorganic membranes are currently available that are resistant to the majority of organic solvents.

Table 2.11: Application of MBR in pharmaceutical and biomedical treatments (12)

Reaction	Membrane reactor	Purpose
Conversion of fumaric acid to L-aspartic acid (*Escherichia coli* with aspartase)	Entrapment in polyacrylamide gel	Pharmaceuticals and feed additives
Conversion of L-aspartic acid to L-alanine (*Pseudomonas dacunhae*)	Entrapment in polyacrylamide gel	Pharmaceuticals
Conversion of cortexolone to hydrocortisone and prednisolone (*Curvularia lunata/Candida simplex*)	Entrapment in polyacrylamide gel	Production of steroids
Conversion of acetyl-D,L-amino acid to L-amino acid (aminoacylase)	Ionic binding to DEAE-sephadex	Production of L-amino acids for pharmaceutical use
Synthesis of tyrosine from phenol, pyruvate and ammonia (tyrosinase)	Entrapment in cellulose triacetate membrane	Production of L-amino acids for pharmaceutical use
Hydrolysis of a cyano-ester to ibuprofen (lipase)	Entrapment in biphasic hollow fibre reactor	Production of anti-inflammatories
Production of ampicillin and amoxycillin (penicillin amidase)	Entrapment in cellulose triacetate fibers	Production of antibiotics
Hydrolysis of a diltiazem precursor (lipase)	Entrapment in biphasic hollow fibre reactor	Production of calcium-channel blocker
Hydrolysis of 5-p-HP-hydantoine to D-p-HP-glycine (hydantoinase and carbamylase)	Entrapment in UF polysulfone membrane	Intermediate for the production of cephalosporin
Dehydrogenation reactions (NAD(P)H-dependent enzyme systems)	Confination with UF-charged membrane	Production of enantiomeric amino acids
Hydrolysis of DNA to oligonucleotides (DNase)	Gelification on UF capillary membrane	Production of pharmaceutical substances
Hydrolysis of hydrogen peroxide (bovine liver catalase)	Entrapment in cellulose triacetate membrane	Treatment in liver failure
Hydrolysis of whey proteins (trypsin, chymotrypsin)	Polysulfone UF membrane	Production of peptides for medical use
Hydrolysis of arginine and asparagine (arginase and asparaginase)	Entrapment in polyuretane membrane	Care and prevention of leukaemia and cancer

Abbreviation: UF, ultrafiltration.

(Giorno et.al, Biocatalytic membrane reactors: applications and perspectives, TIBTECH AUGUST 2000 (Vol. 18), 339-349)

2.7 Recent Development of Biocatalytic Membrane Reactor (BMR)

In recent years, the functions of the membrane have been extended with the systematic use of these reactors in two-phase bioconversions. Membrane acts as a support for the interface between two distinct liquid phases. The membrane not only separates the phases, but also provides interfacial contact area and, together with the enzyme, acts as an interfacial catalyst. A complete retention of the enzyme within the system is the first and most important requirement for a

successful continuous operation of an enzymatic membrane bioreactor. Upon this retention, the enzyme becomes confined to a defined region of the membrane reactor, where reaction with the substrate occurs. A more recent application of membrane bioreactor in the food sector is promoted by the industrial demand in the production of functional food and nutraceuticals, and alternative food or food ingredients that has health benefit. Other applications such as the production of L-aspartic acid (Chibata 1974), dipeptide AspartameTM (Carasik 1983), the production of L-alanine (Oyama 1991), the production of fructose-concentrated syrups (Klibanov 1990) has also been studied.

Currently, research interests are devoted toward (1) different configurations of membranes reactor for biomass separation ,(2) use of low cost membrane materials in membrane bioreactor (MBR) systems, and (3) new operating strategies and design of systems to minimize fouling of membranes in MBR systems. The development of submerged configuration is also emerging in other applications such as biofuel, pharmaceutical, food, and biotechnology. In most of the SMBR system the membrane reactors can be used to carry out the reaction with the substrate and simultaneous separation and purification of the product. Other advanced membrane operations, like microfiltration (MF), UF, nanofiltration (NF), are combined with the MBR system can promote the final concentration and formulation of the products in the line of process intensification strategies. The integration of membrane operations will be among the key strategies to enhance processes precision and efficiency, with maximization of

mass and energy conversion into valuable products while preventing and minimizing waste production.

The development of submerged enzymatic membrane bioreactor (SEMBR) needs a lot of research in order to optimize all the operating conditions for the best possible and efficient solution for different production lines. This thesis intends to develop the SEMBR with different parameters optimized with a new line of approach as well as study of side stream module. Also recovery of value added components from the waste biomass will be an approach with the application of this submerged system. By application of this system we can recover and produce the value added components from the waste biomass (such as agricultural wastes, industrial organic wastes, waste cooking oil etc).

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Chapter 3

Material and Methods

3.1 Chemicals

Lipase from *Candida rugosa* (CRL, E.C.3.1.1.3, MW 67 kDa) was purchased from Sigma Aldrich and had the following characteristics: 1 g protein per 5.88 g solids, 819 units/mg protein. The enzyme solutions were partially purified by centrifugation. All other chemicals were of analytical grade and used without further purification.

Bovine serum albumin (BSA) were purchased from Sigma Aldrich and was used as standard for the protein calibration curve. BCA test from PIERCE was used to measure protein concentration. For preparation of buffer we used di-sodium hydrogen phosphate anhydrous (Na_2HPO_4 ,MW141.96 g/mol) from Fluka Biochemika and sodium phosphate monobasic anhydrous (NaH_2PO_4, MW 120 g/mol) from Sigma Aldrich. Sodiun Hydroxide (NaOH) from Sigma- Aldrich was used as base for the titration of acids produced during reaction. Ultrapure water was obtained from a PURELAB Classic instrument.

Triglycerides present in vegetable oil, such as olive oil, sunflower oil, corn oil, soya oil etc.

Hollow fibre ultrafiltration polyethersulphone (PES) membranes kindly provided by Gambro (Germany) and capillary ultrafiltration polyamide (PA) membranes kindly provided by Berghof (Germany) were used as support for the enzyme immobilization. The membranes had hydrophilic properties and asymmetric structure with the thin dense layer on the lumen side and the sponge layer on the shell side. The nominal molecular weight cut-off (NMWCO) of the selective layer was 50 kDa.

The PES membranes had an inner outer diameter of 215 µm-265 µm, respectively; the PA capillaries inner/outer diameter was 1.2/1.4 mm, respectively. Both membranes are stable to organic solvent and to acids and alkalies in a pH range 3 and 12 and thermal resistance until 80 ˚C.

Both membranes have been used to prepare side stream and submerged modules. However, the final selection of PES hollow fiber membranes for the development of submerged membrane system was made on the basis of the fact that their smaller diameter allowed higher resistance to mechanical distorsion in the module U-shape.

3.2. Reaction equation and bi-phase system

The hydrolysis of triglycerides present in vegetable oil, such as olive oil, to fatty acids and glicerol (**Fig 3.1**) was used as a model reaction for the development of biocatalytic membrane systems.

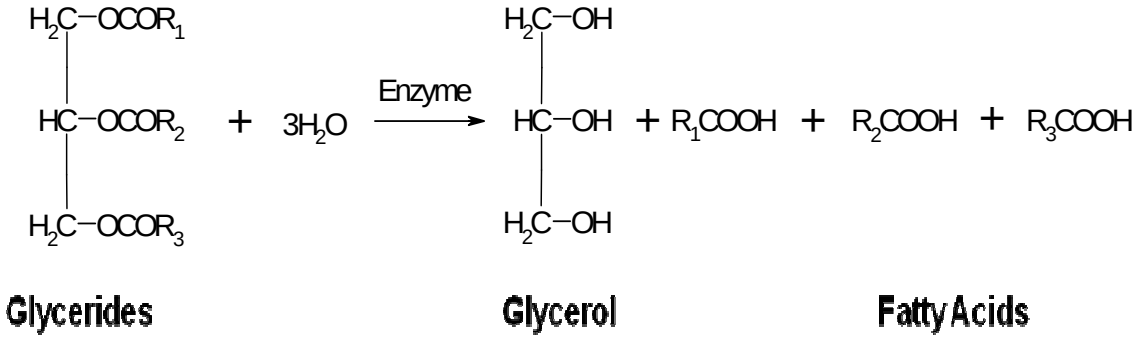

Fig: 3.1 The schematic of two phase system with extraction of reaction product

The biphase membrane system (**Fig. 3.2**) is constructed in such a way that the enzyme is immobilized within the membrane; the oil phase is located from one of the two side of the membrane (the one most close to the immobilized enzyme) and the aqueous phase is located from the other one.

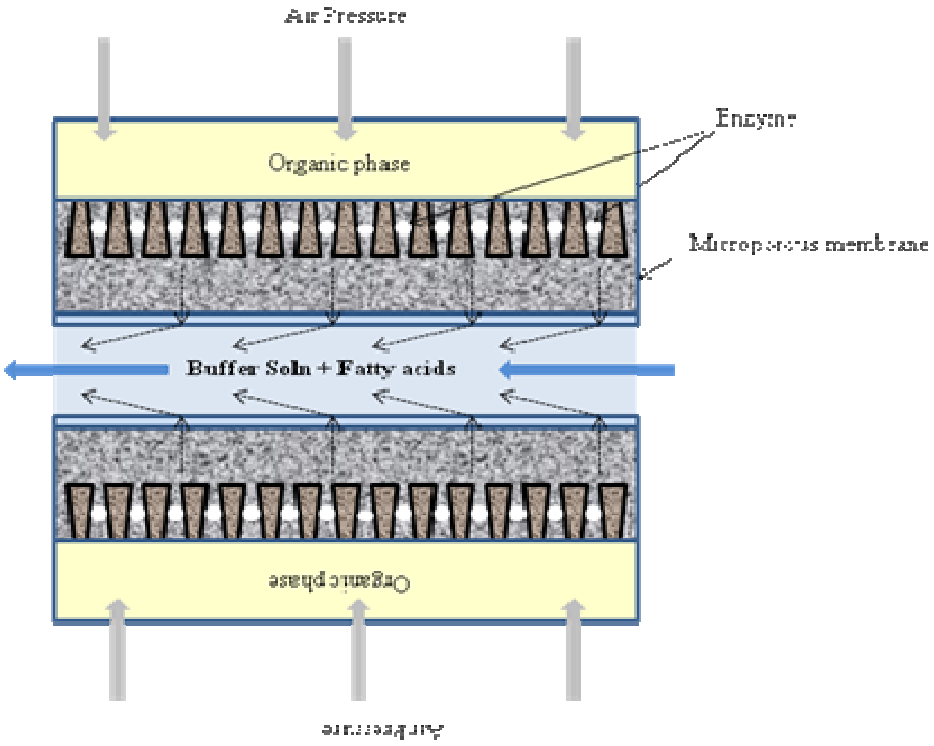

Fig. 3.2. Schematic representation of bi-phase membrane reaction system

This conformation allows the triglycerides to diffuse through the membrane reaching the immobilized enzyme that will catalyze their hydrolysis and then the formed fatty acids and glycerol will be extracted into the aqueous phase. The bi-phase system allows the simultaneous reaction and product separation. The gradient concentration promotes the mass transfer by diffusion and component solubility in different phases promotes the separation. Enzyme-loaded membranes

work as a catalytic contactor/separator at which level the two phases are in contact and at the same time separated (they are not dispersed into each other).

In this work, the two phase system was realized using membranes in ”side stream” (i.e. the membranes are located esternally to the bulk and the feed is supplied by means of a pump) and ”submerged” configuration (i.e. the membranes are immersed within the bulk to be treated).

3.3 Equipments and operation mode

3.3.1. Preparation of Modules

The side stream membrane module has been prepared with the help of a glass tube or shell (**Fig:3.3**) where the membrane has been encased and fixed with epoxy resin. **Table : 3.1** describes the properties of the side stream configuration using polyamide capillary membranes.

Table : 3.1 Specifications of the PA capillary membranes

Membrane material	PA
Nominal pore size(kDa)	50
Fiber length (mm)	120
External diameter (mm)	1.2
Internal diameter (mm)	1.4
No. of membranes in module	4
Total membrane surface area (m^2)	2.3×10^{-3}

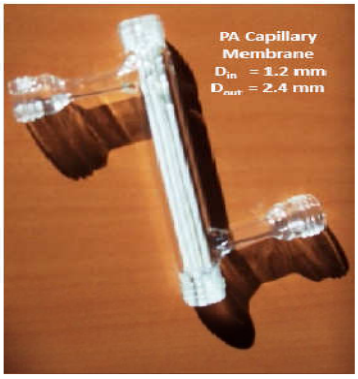

Fig 3.3: Side stream Module

The submerged modules usign polyethersulphone membranes is illustrated in **Fig 3.4** and its properties are summarized in **Table 3.2**.

Fig 3.4: Submerged Module

Table 3.2. Specifications of the PES hollow fibre membrane

Membrane material	PES
Nominal pore size (kDa)	50
Fiber length (mm)	110
External diameter (μm)	265
Internal diameter (μm)	215
No. of membranes in module	15
Total membrane surface area (m^2)	2.75×10^{-3}

The membrane surface area has been calculated considering the effective lenght (i.e., overall lenght excluding the length fixed with epoxy resin):

$$A = 2\pi rh * n$$

Where A is the total surface area, r is the radius of the cross section of the membrane, h is the effective length of the membranes used, n is the number of fibres used.

3.4 Membrane Characterization

The pure water permeability is defined as the volume of water that passes through a membrane per unit time, per unit area and per unit of transmembrane pressure. All the membrane modules both side stream and submerged have been

characterized before immobilization and reaction using distilled ultrapure water (Fig 3.5). The detail of the characterization results for both of the module are shown later on in Chapter 4.). The permeate flux in time as a function of different trans membrane pressure values was measured; the steady-state values of flux were then plotted in respect to TMP and, from the slope of the straight line obtained, the permeability was calculated. From Darcy law $J = L_P.\Delta P$ here *J* is the permeate flux in liters per hour per square meter, *Lp* is the permeability in liters per square meter per hour per bar, ΔP is the trans membrane pressure in bar. The membrane pure water permeability was about 300 L/m2*h*bar for the polyamide membranes. The pure water flux for the submerged module has also been determined with the same concept..The reason for measuring this parameter was to have the baseline for the membrane performance and monitor it in subsequent uses. In addition, the reduction of permeability after enzyme immobilization was an indirect evidence of the stable presence of the enzyme within the membrane. The permeability of membranes containing the enzyme decreased as a function of amount of immobilized enzyme.

3.5 Enzyme immobilization

Lipase solution

The lipase solution were prepared by dissolving the raw lipase in 50 mM phosphate buffer of different pH (6.0 to 9.5) respectively and gently stirring for about two hours at very low rpm (150-200) at the room temperature. Afterwards the solution was centrifuged for 15 minutes at 3000 RPM in a centrifuge machine.

The undissolved part was removed. The final solution of a sepcific amount were then pressed through the membrane from shell to lumen and the enzyme was entrapped into the porous layer of the sponge layer of the PES HF or PA membrane due to different MWCO.

The protein content of enzyme solution was determined spectrophotometrically using the kit produced by Sigma Chemicals (Cat. No: P 5656). The Lowry procedure has been found to be one of the most reliable and satisfactory method for quantifying soluble proteins. The procedure described here is based on Peterson's modification of the Micro-Lowry method (Peterson, 1977) and the method of Lowry et al (1951). Reagents used are Lowry and Folin & Ciocalteu's Phenol reagent while protein standard are prepared from Bovine Serum Albumine, Fraction V [BSA].

This method is based on the principle that an alkaline cupric tartrate reagent complexes with the peptide bonds and forms a purple-blue color when the phenol reagent is added. Absorbance is read at a suitable wavelength between 562nm. The protein concentration is determined from a calibration curve. The following table (Table 3.3) describe about the different standard calibration measurements with the average amount of protein at certain concentration (0.2,0.4,0.6,0.8,1.0) respectively. In the table only a few has been described . In different module and in different initial concentration the amount of absorbed protein is almost same.

Table 3.3: Standard measurement of BSA protein

	[BSA] (mg/ml)	ABS (nm)	ABS (nm)	ABS (nm)	ABS (nm)	Stdev
Blank	0	0	0			0
BSA	0.2	0.256	0.249	0.283	0.289	0.019704
BSA	0.4	0.454	0.489	0.532	0.476	0.032847
BSA	0.6	0.663	0.667	0.728	0.698	0.030342
BSA	0.8	0.827	0.848	0.909	0.919	0.045177
BSA	1	0.956	1.012	1.101	0.992	0.061684

The absorbance values of the standards were plotted versus their protein concentrations to prepare a calibration curve .The protein concentrations of the other samples were determined from the calibration curve, and the result was multiplied by the appropriate dilution factor to obtain the protein concentration in the original sample.

3.6 Immobilization Set Up and Procedure

3.6.1 Enzyme immobilization in side stream module

In the side stream configuration (**Fig: 3.5)**, the enzyme has been immobilized by cross-flow ultrafiltration. The enzyme solution is pressed from the shell side to lumen by applying a transmembrane pressure, using a peristaltic pump. The permeate has been collected from the lumen side.

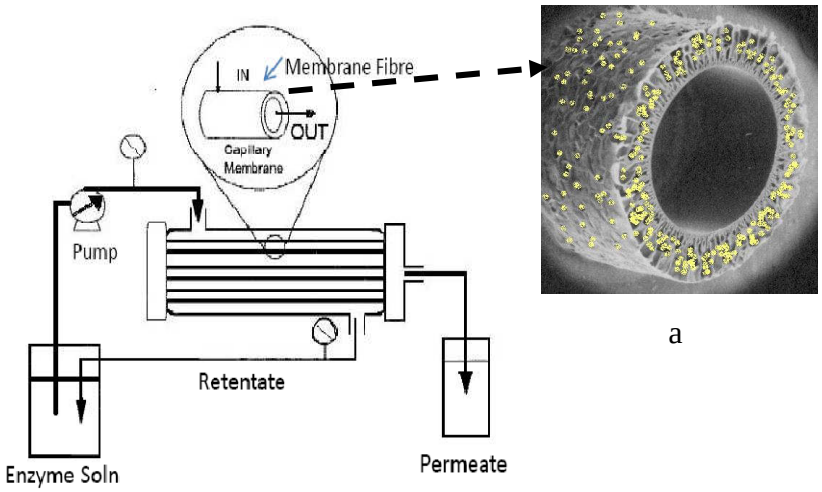

Fig: 3.5 Immobilization system for the side stream module

400 mL of 3 g/L enzyme solution were recirculated along the shell side at a flow rate of 120 mL/min, with a trans membrane pressure of 0.3 bar. In this way, the lipase (MW 67 kDa) entered the spongy layer but could not pass through the thin layer (cut-off 50 kDa) because of the relative size. The enzyme was immobilized throughout the membrane tickness and along the membrane length (**Fig. 3.5a**).

During immobilization, the decrease of the permeate flux indicated the accumulation of enzyme within the membrane. Once the flux did not decrease anymore, the enzyme solution was removed and the circuit was rinsed with distilled and prefiltered water as long as no additional protein was detected (by measuring optical density at spectrophotometer).

Then again we measure the pure water permeability to verify the entrapment of enzyme in the shell side of the membrane. The decrease of pure water permeability gives an indication of the enzyme present in the membrane.

The mas of immobilized enzyme is measured by BCA test and calculated by mass balance between initial and final samples.

3.6.2 Enzyme immobilization in submerged module

For immobilization in submerged condition nitrogen gas was used for pressurizing the lipase solution from the shell side. Manometers are used to regulate the pressure from both side of the membrane system. A thermostatic bath was used to keep the temperature constant of the tank. Magnetic stirrers are used to stirr the tank solution of aqueous phase. During immobilization the tank contained the enzyme solution (**Fig 3.6**) has been connected with the module . The applied pressure promoted permeation of water through them, while enzyme was entrapped within the spongy layer and could not pass through the pores in membrane layer due to its large size compared to the membrane pore size. The other end of the vessel is also kept closed during immobilization.At the end of the process, the system was rinsed in order to remove the non stable entrapped enzyme in the surface of the membrane.

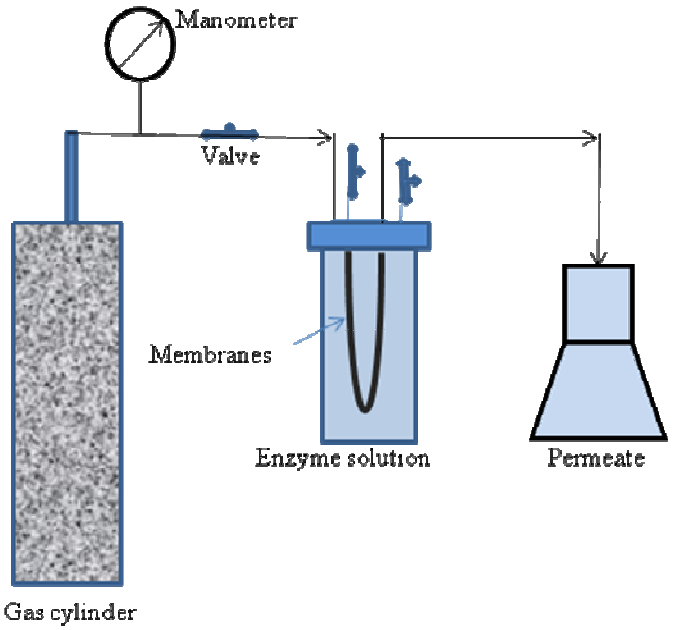

Fig 3.6: Immobilization system for submerged module

3.7 Reaction Procedure in Bi-phase system in Side stream Module

The membrane contained the lipase immobilized within the spongy layer. The organic phase (organic oil) was circulated along the shell side and the aqueous phase (250 ml of phosphate buffer, pH 7) was circulated along the lumen side. Gear pumps were employed to supply the organic and aqueous phases to the reactor. Two control panels, with valves, flow meters and pressure gauges, were used to control, separately, the shell and lumen circuits (Sepracor Inc., MA, USA) (**Fig 3.7**). In the aqueous phase a pH stat is attached to monitor the acid generation in the aqueous phase and keep the pH constant at 7 (unless otherwise specified) by adding base (Sodium Hydroxide). The system was kept at room temperature (26±1 ˚C).The immobilized enzyme was stably entrapped within the membrane, it was not removed into the aqueous phase (lumen side) , as well as it

did not diffuse into the organic phases (shell side) since it was not soluble in pure organic solvent.

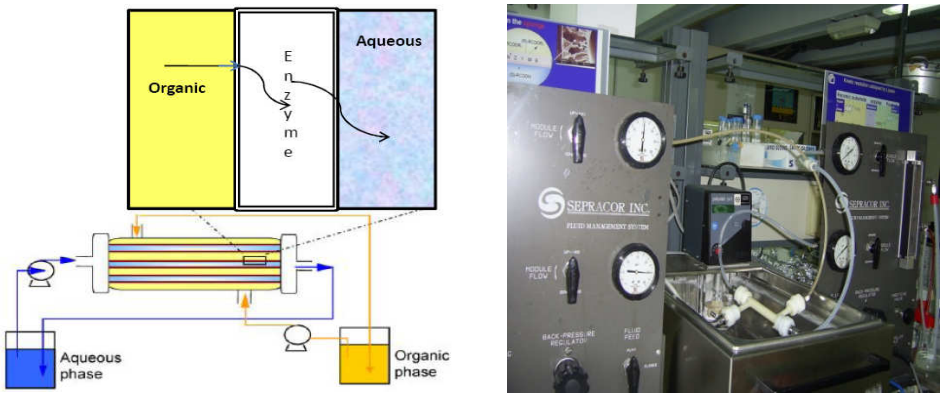

Fig 3.7: Two phase system for side stream reaction

3.8 Reaction Procedure in Bi-phase system in Submerged configuration

After the immobilization the reaction with organic substrate has been performed. In the reraction tank there are the organic phase in the shell side of the membrane and a phosphate buffer was circulated throught the lumen side. A certain TMP has been applied all the cases in order to avoide the displacement of organic phase into the aqueous phase or vice versa. The Reaction has been carried out primarily with commercial grade olive oil (acidity < 1%) and then same fried oil, after washing the module with the phosphate buffer several times, removing the surface enzyme from the membranes. The reaction configuration is described in **Fig. 3.8** where a manometer have been connected at the inlet of pressurize unit with a regulate valve but the other end kept closed. The organic phase has been in the shell side of the membrane in contact with the enzyme at the interface of two liquid phases. Phosphate buffer solution has been circulated through the lumen

side by a gear pump. Two manometers have been connected with inlet and outlet of aqueous phase in order to maintain a constant TMP during the reaction. Very low axial flow rate has been applied to maintain the low pressure drop.

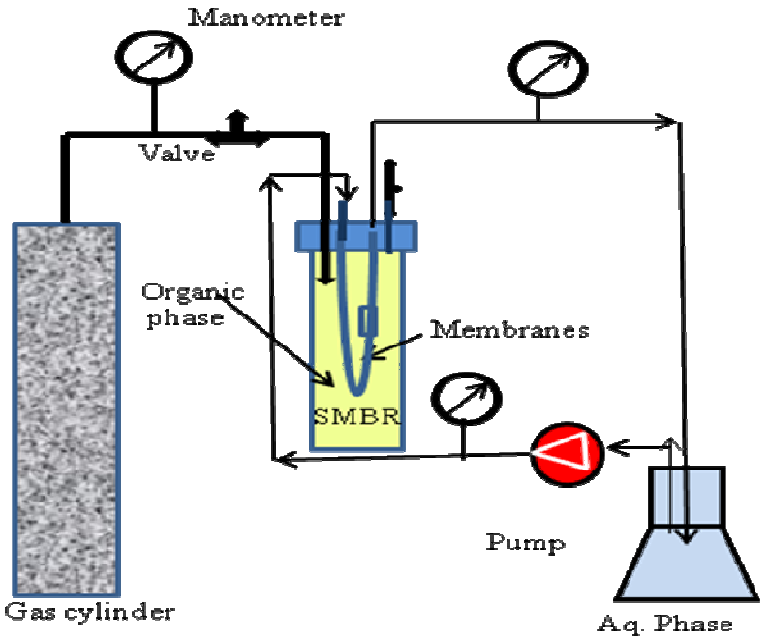

Fig 3.8 : Submerged Reaction System

3.9 Reaction rate measurement

The reaction rate has been monitored by titration of the acids extracted into the aqueous phase (50 mM phosphate buffer (Na_2HPO_4/NaH_3PO_4). An automatic titrator (Mettler DL 25) loaded with a 20mM NaOH solution was used. The titrator continuously adds base to titrate the formed acids. The mol of base are equivalent to the mol of acids produced. Therefore, from the volume of the added base, knowing the base concentration, it was possible to calculate the mol of acids as a function of time.

In **Table 3.4** an example of how the data have been callected and elaborated is illustrated.

Table 3.4 Example of titration table and calculations

a	***b***	***c***	***d***	***f***
Time (min)	Volume of base added (ml)	µmol of base equivalent to umol of acids produced= (***b*** *20 mM)	Initial volume of reaction Tank(aqueous Phase)	Amt of Acid ***(c/d)***(mmol/l)
0	0.000	0.0	200	0.0
15	1.695	33.9	200	169.50
30	3.685	73.7	200	368.50
45	8.015	160.3	200	801.50
60	8.764	175.2	200	876.49
75	10.255	205.1	200	1025.50
90	11.765	235.3	200	1176.50
105	12.98	259.6	200	1298.00
120	14.07	281.4	200	1407.00
135	14.895	297.9	200	1489.50
150	15.965	319.3	200	1596.50
165	17.404	348.08	200	1740.40
180	18.134	362.68	200	1813.40
195	18.614	372.28	200	1861.40
210	19.269	385.38	200	1926.90
225	19.914	398.28	200	1991.40
240	20.254	405.08	200	2025.40
255	20.904	418.08	200	2090.40
270	21.439	428.78	200	2143.90
285	22.064	441.28	200	2206.40
300	22.329	446.58	200	2232.90

Base NaOH = 20 mM

Each series of experiments was repeated at least three times and the standard deviation was calculated according to the equ. 3.1 where S is the measured standard deviation, N is the number of experiment done, $\overline{X}$ is the arithmetic mean of the sample.

$$S = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(X_i - \overline{X})^2} \quad \text{.....} \qquad \text{Equ 3.1}$$

The error associated to the experiments was evaluated. The experimental overall error of 7% was associated to the experiments (equ 3.2) measured by following equation.

$$SE\bar{x} = \frac{S}{\sqrt{n}} \times 100 \text{.....} \quad \text{Equ 3.2}$$

Where S is the sample standard deviation (i.e., the sample based estimate of the standard deviation of the population), and *n* is the number of observations of the sample.

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Chapter 4

Results and Discussions

The catalytic activity of lipase immobilized in the submerged system was studied at various operating conditions such as pH, T and fluid dynamics parameters. The optimization study is presented in this chapter. The optimal conditions were then used to test the reactor efficiency with fried vegetable oil.

4.1 Membrane Characterization

Membrane modules were characterized by measuring the pure water permeability. The permeate flux as a function of time for different transmembrane pressure values was measured; the steady-state values of flux were then plotted versus TMP and, from the slope of the straight line obtained, the permeability was calculated (as illustrate in the Materials and methods chapter). The pure water permeability provides an easy way set the initial performance of a membrane and to monitor it for subsequent uses. **Fig. 4.1** and **4.2** illustrate examples of the general flux behavior as a function of time for the polyamide and polyethersulphone membranes, respectively.

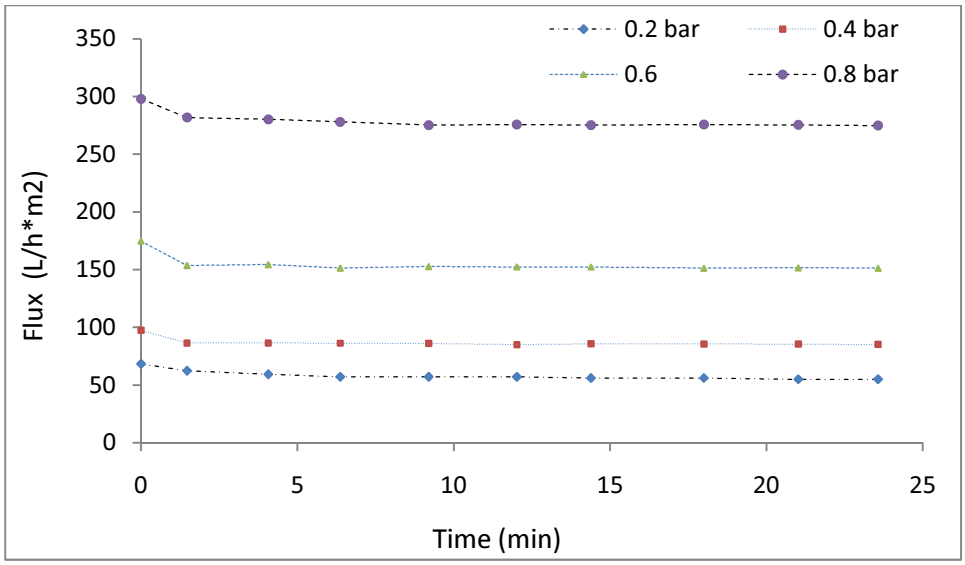

Fig. 4.1: Permeate flux as a function of time at different transmembrane pressure for polyamide membranes in side stream module configuration

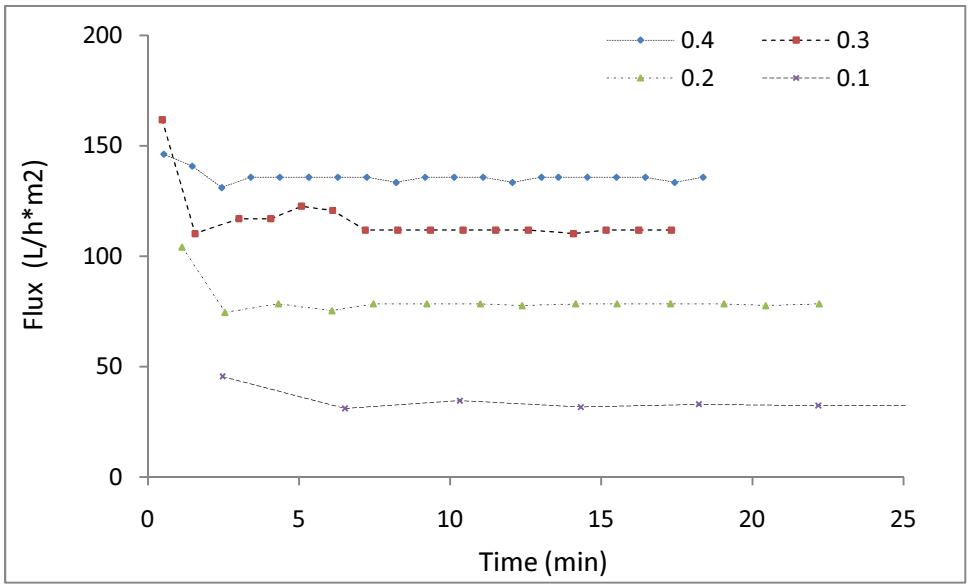

Fig 4.2: Permeate flux as a function of time at different TMP for polyethersulfone membranes in submerged module configuration

From these type of graphs, the initial pure water permeability has been evaluated for both polyamide **(Fig 4.3)** and polyethersulfone (**Fig 4.4**) membranes. In each case, a comparison with the pure water permeability after immobilization is illustrated. The

reduction of permeability is an evidence of the presence of the enzyme within the membrane.

It can be seen that, although both membranes have same molecular weight cut-off, the initial pure water permeability of polyamide membranes is slightly higher. This can be due to difference in porosity (pores area /membrane surface area) and to slightly different hydrophilic character.

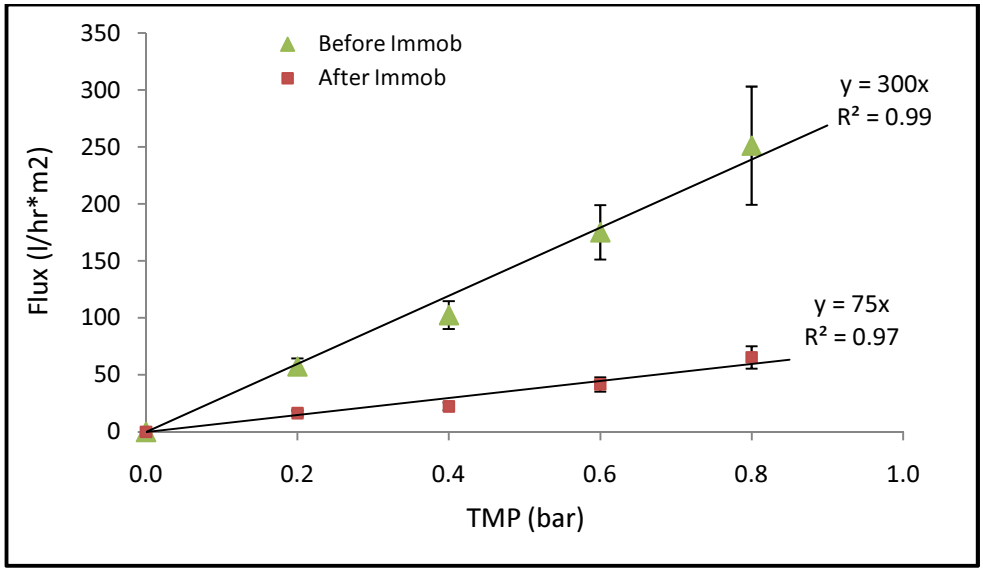

Fig 4.3: Pure water Permeability of polyamide membranes in side stream configuration

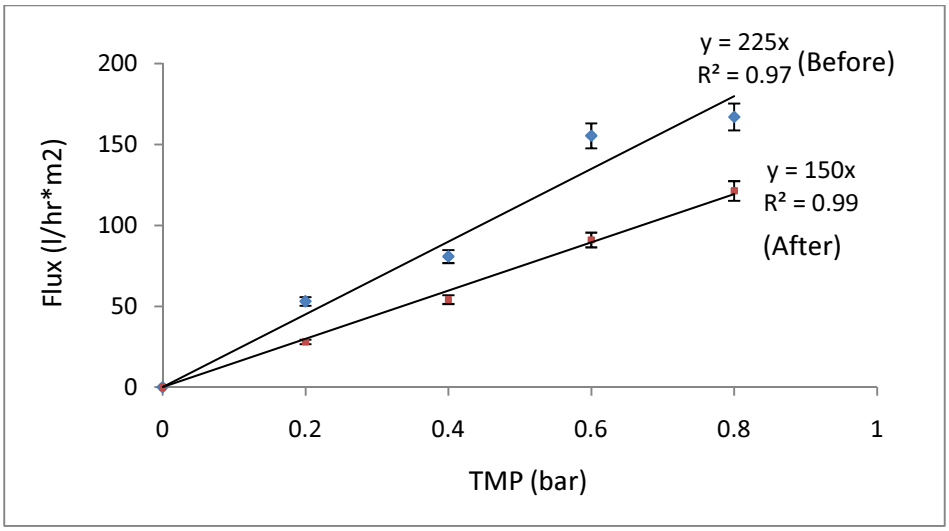

Fig 4.4: Pure water Permeability of polysulfone membranes in submerged configuration.

4.2 Immobilization

Enzyme has been immobilized by cross-flow ultrafiltration in side stream modules and by dead-end ultrafiltration in submerged modules.

For the side stream module, 400 mL of 3 g/L *Candida rugosa* enzyme solutions in phosphate buffer were used. The solution was recirculated along the shell side at a flow rate of 120 mL/min, with a transmembrane pressure of 0.3 bar.

For the submerged module, 50 mL of 3g/L *Candida rugosa* enzyme solutions in phosphate buffer were used. The solution was pressurized at 0.3 bar by gas cylinder.

Figs 4.5 and 4.6 illustrate the general behavior of water flux as a function of time during immobilization procedure for side stream and submerged configuration modules. The overall effects on pure water permeability have been presented in Figs 4.3. and 4.4, respectively.

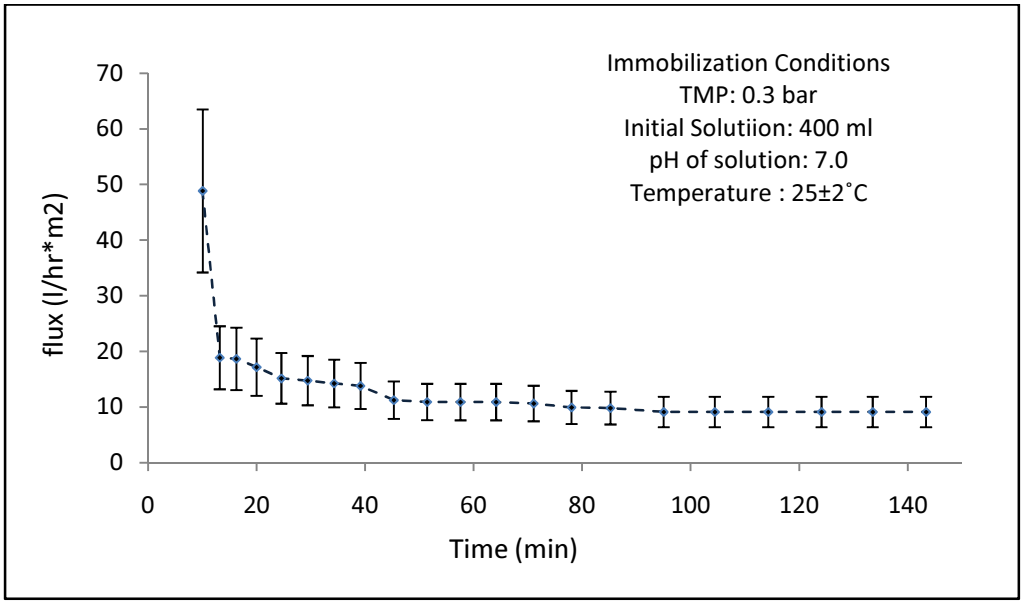

Fig 4.5: Flux behavior as a function of time during enzyme immobilization by cross-flow filtration in side stream module

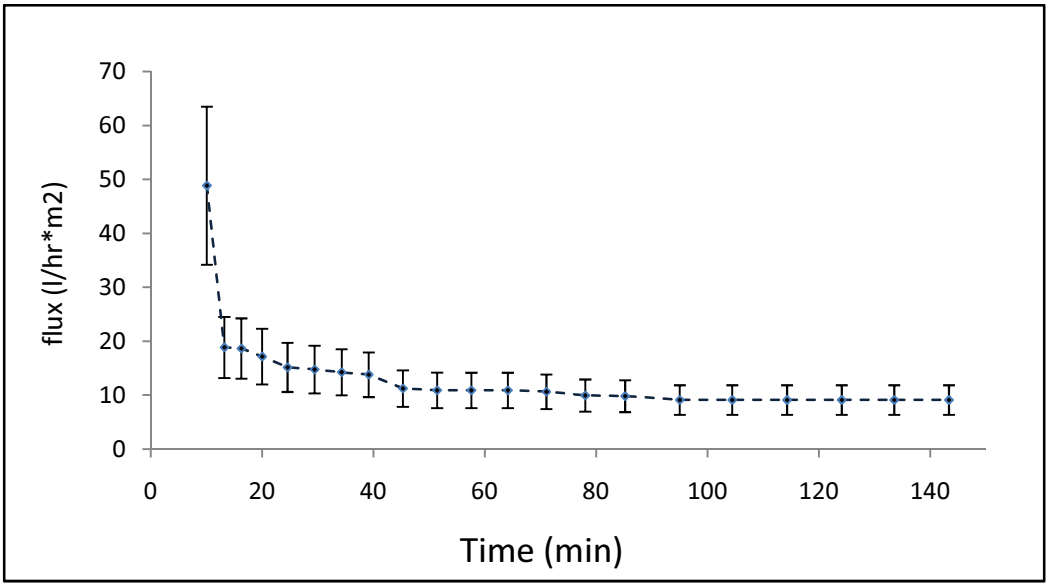

Fig 4.6: Flux behavior as a function of time during enzyme immobilization by dead-end filtration in submerged module

The protein concentration has been measured in initial, permeate, retentate and rinsing solutions by BCA test (see Materials and method chapter). The immobilized protein mass has been estimated by mass balance between the measured protein solutions. The amount of immobilized enzyme for various side streams (Fig. 4.7) and submerged (Fig. 4.8) membrane modules is reported.

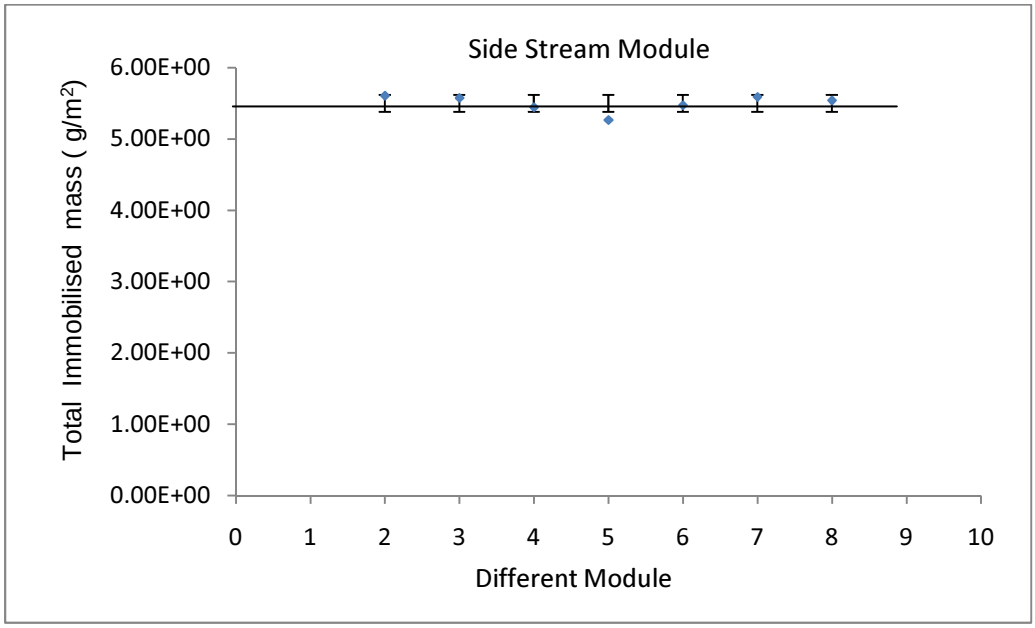

Fig 4.7: Amount of Immobilized enzyme in different side stream module

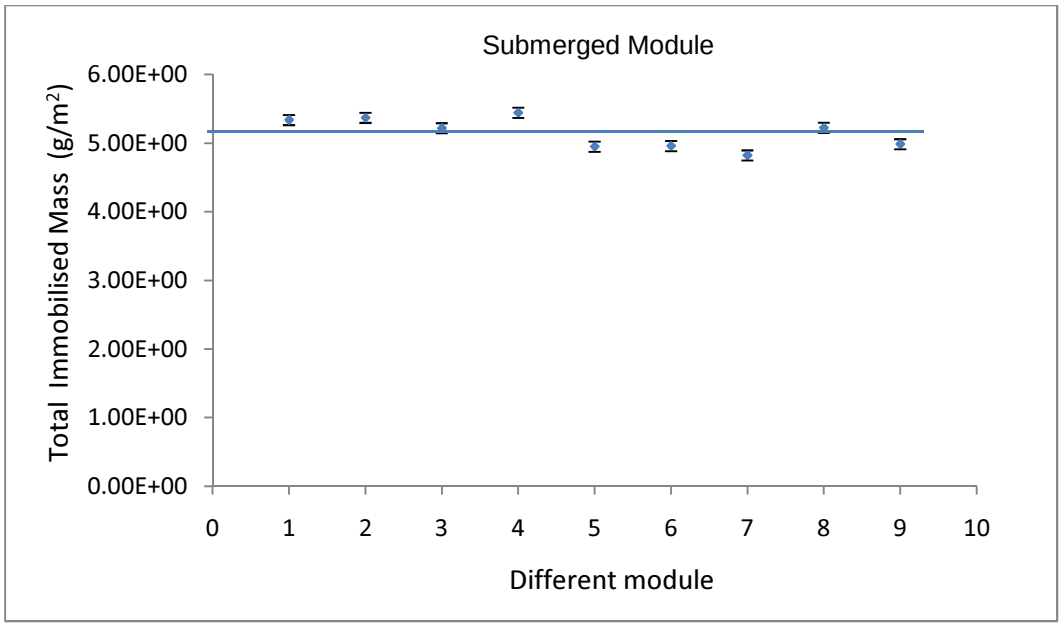

Fig 4.8: Amount of Immobilized enzyme in different submerged module

The average amount of immobilized enzyme per membrane surface area in side stream module was around 5.5 (± 0.3) g/m^2 and in submerged modules was 5.2 (±0.3) g/m^2

4.3 Reaction in Bi-phase system

As illustrated in Fig. 3.6, the bi-phase system is constructed in such a way that the membrane containing the lipase immobilized within the spongy layer in the shell side is placed in contact with the organic phase (vegetable oil) while the aqueous phase is located in the lumen side.

For the side stream reaction gear pumps were employed to supply the organic and aqueous phases to the reactor while in the submerged configuration the organic phase was located within the tank containing the membrane (which was

pressurized by a gas cylinder) and the aqueous phase was circulated along the lumen by a pump (see equipment paragraph in previous chapter).

By enzyme activity tests and protein measurements in samples of water and oil phases in contact with the enzyme-loaded membrane for about 24 hours, it was verified that the immobilized enzyme was stably entrapped within the membrane, it was not removed into the aqueous phase (lumen side) since its molecular size (67 kDa) was larger than the membrane molecular weight cut-off (50 kDa), as well as it did not diffuse into the organic phases (shell side) since it was not soluble in pure organic solvent.

The reactor performance was evaluated by measuring the increase of product concentration into the aqueous phase, from which the observed volumetric reaction rate was calculated. The observed reaction rate is different from the intrinsic reaction rate, since it is calculated from the product concentration in the bulk solution (P_b, i.e. the product diffused into the bulk from the reaction site within the membrane, see reaction equation 1.4). Anyway, it is a convenient and reliable way to verify the reactor performance.

An example of product concentration behavior in water phase as a function of time is reported in Fig. 4.9 and Fig. 4.10 for side stream and submerged configuration, respectively.

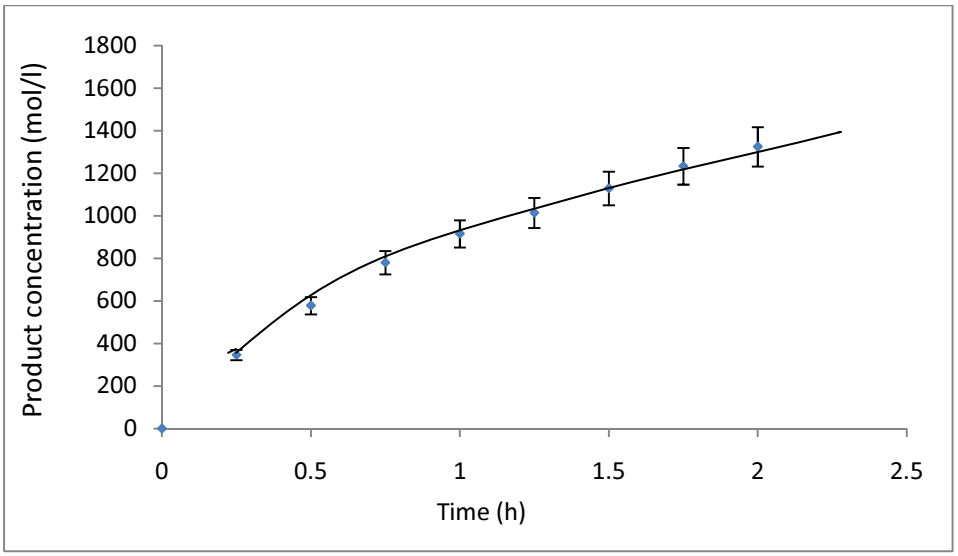

Fig. 4.9: Behavior of fatty acids concentration in the aqueous phase as a function of time using the enzyme-loaded membranes in side stream module

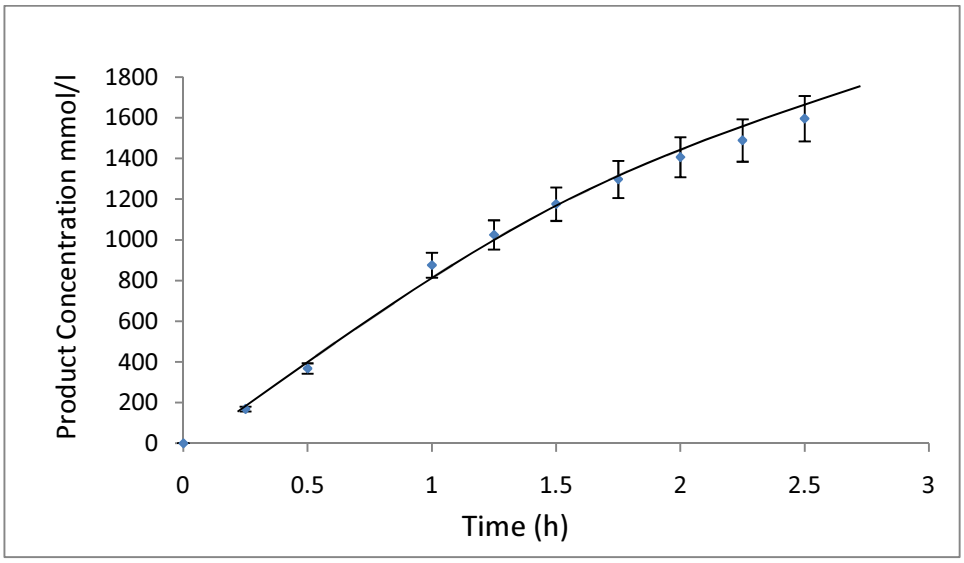

Fig. 4.10 : Behavior of fatty acids concentration in the aqueous phase as a function of time using the enzyme-loaded membranes in submerged module.

From these types of graphs, the reaction rate has been calculated as the tangent to the curve in the initial linear part. For side stream and submerged reactors, the

average value of reaction rate related to the reported average mass of immobilized enzyme, was 850 (± 50) mmol/l h.

4.4 Optimization of Submerged Biocatalytic Membrane Reactor

In this section the results of the optimization study of the unconventional submerged biocatalytic membrane reactor (SBMR) is discussed.

4.4.1 Influence of pH

The effect of buffer pH on lipase activity in biphasic SBMR with lipase immobilized has been carried out. A comparison with the free enzyme activity is also reported.

For the bi-phase system, the amount of immobilized enzyme was about 13.7 mg, the membrane area was 2.75×10^{-3} m^2. The hydrolysis of triglycerides from olive oil was performed in the pH range of 6.0 to 9.0. Six different modules have been used and for each one several experiments series have been repeated in various pH values as summarized in **Table 4.1**.

Table 4.1: Study of different module in different pH conditions with immobilized enzyme

Module.No	pH 6	pH 6.5	pH 7	pH 7.5	pH 8	pH 8.5	pH 9
	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1
	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2
Module 1	Exp#3	Exp#3	Exp#3	Exp#3	Exp#3	Exp#3	Exp#3
	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1
	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2
Module 2	Exp#3	Exp#3	Exp#3	Exp#3	Exp#3	Exp#3	Exp#3
	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1
	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2
Module 3	Exp#3	Exp#3	Exp#3	Exp#3	Exp#3	Exp#3	Exp#3
	Exp#1	Exp#1	Exp#1	Exp#1			
	Exp#2	Exp#2	Exp#2	Exp#2		-----	-----
Module 4	Exp#3	Exp#3	Exp#3	Exp#3	-----		
	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1
	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2
Module 5	Exp#3	Exp#3	Exp#3	Exp#3	Exp#3	Exp#3	Exp#3
	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1
	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2
Module 6	Exp#3	Exp#3	Exp#3	Exp#3	Exp#3	Exp#3	Exp#3

The free enzyme has been studied in batch, in a stirred tank reactor using 19 ml of phosphate buffer of different pH, 1ml of olive oil, 1 ml of enzyme solution (containing 3 mg of protein) at 590 rpm & 35 ˚C temperature. The series of experiments carried out with free enzyme are summarized in **Table 4.2**.

Table 4.2: Study of different module in different pH conditions with free enzyme

Test .No	pH 6	pH 6.5	pH 7	pH 7.5	pH 8	pH 8.5	pH 9
Test #1	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1
	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2
Test #2	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1
	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2
Twst#3	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1	Exp#1
	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2	Exp#2

From each series of experiments at various pH operating conditions, the average production formation rate value, the related standard deviation and percentage

error has been calculated and summarized in Fig. 4.11 and Fig 4.12. An overall error of 9% resulted associated to both systems.

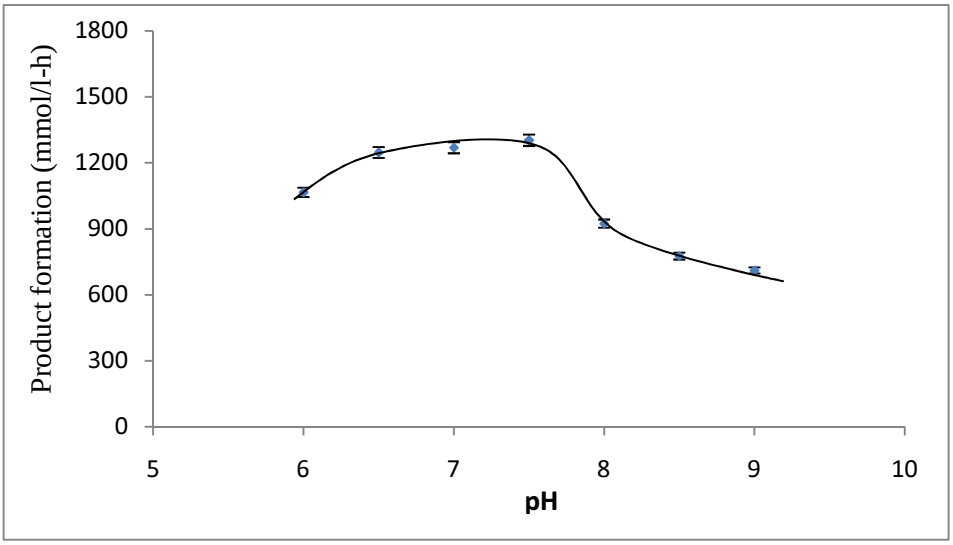

Fig 4.11: Effect of pH on fatty acids production by immobilized lipase, (transmembrane pressure: 0.8 bar; aqueous phase flow rate: 0.06 m/s; organic phase stirring: 850 rpm; temperature: 35 ˚C; olive oil as organic phase).

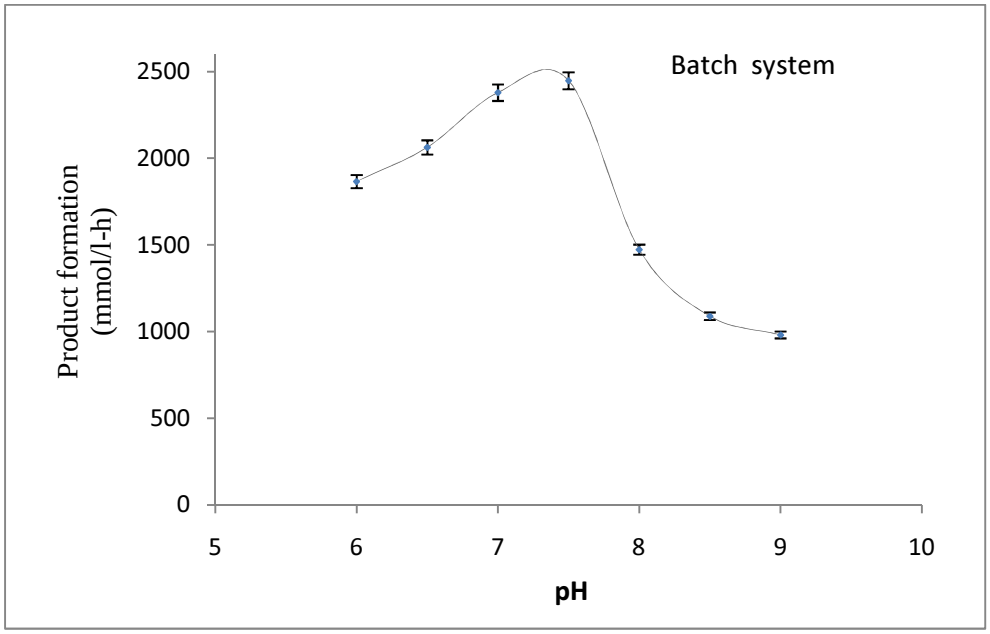

Fig 4.12: Effect of pH on fatty acids production in batch systems with temperature: 35 ˚C; pure olive oil as organic phase 1 ml; 3 mg of enzyme; 19 ml of phosphate buffer of pH 7 stirred at 590 rpm

The optimal pH value resulted 7.5 for both immobilized and free enzyme. From results it can be seen that immobilized enzyme showed a larger range of pH stability compared to the free one. In fact from 6.5 to 7.5 its activity is almost constant, while for the free enzyme the activity is stable from pH 7.0 to 7.5. This observed higher range of pH stability may depend on a higher molecular rigidity promoted by the interaction of the enzyme with the membrane.

It is worth nothing that the production formation rate values at optimal pH conditions for immobilized and free enzyme appear different because the firt is an observed volumetric reaction rate (the enzyme is immobilized within the membrane and the product is measured within the bulk), while the latter is an intrinsic reaction rate (the enzyme is free in a stirred tank reactor). In addition, the amount of enzyme and the reactor volumes are different, therefore a thorough comparison between the two systems is difficult. A rough estimation of the efficiency of immobilized enzyme in comparison with the free enzyme has been tempted on the basis of the enzyme specific activity – $mmol/h \cdot mg_{protein}$ (eventought for the immobilized enzyme is still an observed specific activity). The observed activity of the immobilized enzyme resulted about 10 $mmol/h \cdot mg_{protein}$ while the specific activity of the free enzyme resulted about 15 $mmol/h \cdot mg_{protein}$, i.e. the observed activity of immobilized enzyme was about 66% of the intrinsic specific activity of free enzyme. From literature data (Giorno et al, 2007), it is known that the observed reduction is due to both location of some enzyme molecules far from the o/w interface and to mass trasport rate. The overall stability of the immobilized enzyme was much higher (no decay of

enzyme activity for more that 30 days of oservation) compared to the free one (half-life time of about 15 hours).

4.4.2 Influence of Temperature

Fatty acid production as a function of temperature is shown in Fig 4.13. The behavior of the immobilized enzyme corresponds to the classical one of free enzyme as a function of temperature. Six different module have been studied in all different temperature conditions (**Table 4.3**) from 25˚C to 45 ˚C at an interval of 5˚C. The standard deviation and the percentage of error has been calculated which is around 3%. In **Fig 4.13** each point represents the average of various experiments carried out. It can be seen that the activity increased with increasing temperature and then decreased over 35˚C, which indicated that higher temperature led to the thermal deactivation (Deng et al. 2005). Therefore, the optimum temperature for enzymatic hydrolysis was obtained at 35˚C in these submerged conditions and maintained all over the subsequent experiments.

Table 4.3: Study of different module in different temperature conditions

	Temperature				
Module .No	**25˚C**	**30˚C**	**35˚C**	**40˚C**	**45˚C**
Module 7	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3
Module 8	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3
Module 9	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3
Module 10	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	----	----
Module 11	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3
Module 12	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3

In the literature (Pugazhenthi et. al 2004) it is reported that the lipase activity increases with the increases of temperature which is consistent with the work described here, but it starts decreasing after 40˚C. In that work the enzyme was immobilized on polypropylene microfiltration membrane. The more hydrophobic support might be responsible for the higher temperature range stability of the enzyme. In our work, the selection of the more hydrophilic membranes was done on the basis of its higher stability to organic phase and therefore to higher capacity of the system to keep the two phase in contact within the membrane avoid dispersion of one phase into each other.

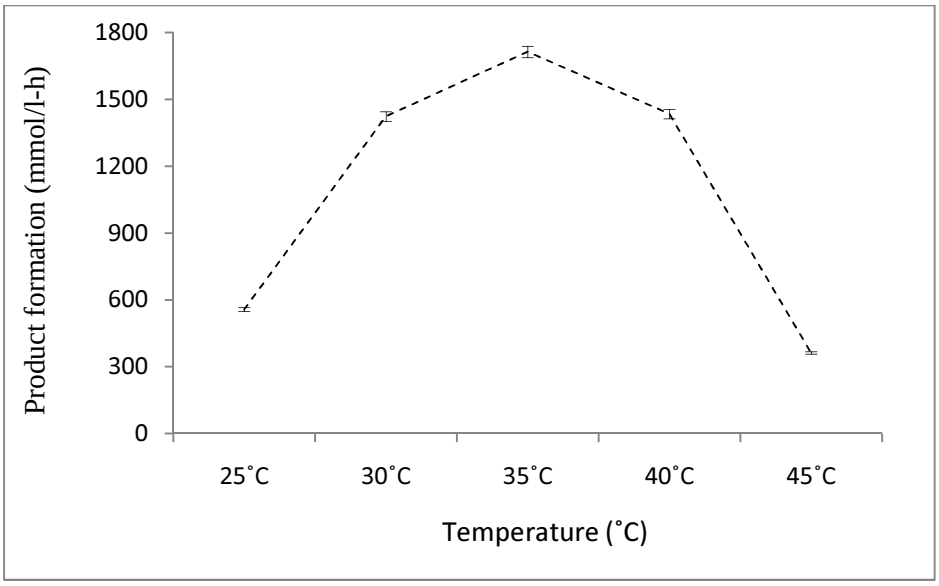

Fig 4.13: Effect of temperature on fatty acids production by immobilized Lipase (enzyme amount: 1.35g/m^2) transmembrane pressure: 0.8 bar; aqueous phase flow rate: 0.06 m/s; organic phase stirring: 850 rpm; pH- 7.0; pure olive oil as organic phase

4.4.3 Influence of Organic Phase Stirring

Experiments have carried out monitoring the reactor performance with organic phase on both static and stirring conditions. The aim was to measure the optimum stirring rate that would allow efficient mass transfer of the substrate through the membrane to the reaction site so that to balance reaction rate with transport rate. Six different module has been studied in all different organic phase stirring rate at 200, 400, 600, 800, 1000, 1200 and 1400 rpm (**Table 4.4**). The standard deviation and the percentage of error has been calculated which is around 6%. As shown in Fig 4.14, 800 to 900 rpm resulted the optimum value range as additional rpm increases resulted in a decrease of reactor performance.

Table 4.4: Study of different module in different organic stirring conditions

Organic Stirring Rate (RPM)							
Exp.No	**200**	**400**	**600**	**800**	**1000**	**1200**	**1400**
Module 13	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3
Module 14	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3
Module 15	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3
Module 16	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	----	----	-----	-----	-----
Module 17	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3
Module 18	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3

The reason for this observed behavior may rely on the fact that, over the mentioned rpm value, the higher transport of substrate to the enzyme obtained at higher organic phase flow rate caused substrate inhibition phenomena (Molinari 1997, Giorno 1997). A confirmation of this effect could be obtained by the fact that, reducing the rpm at the optimum indicated range, the reactor performance returned to be the previously observed one.

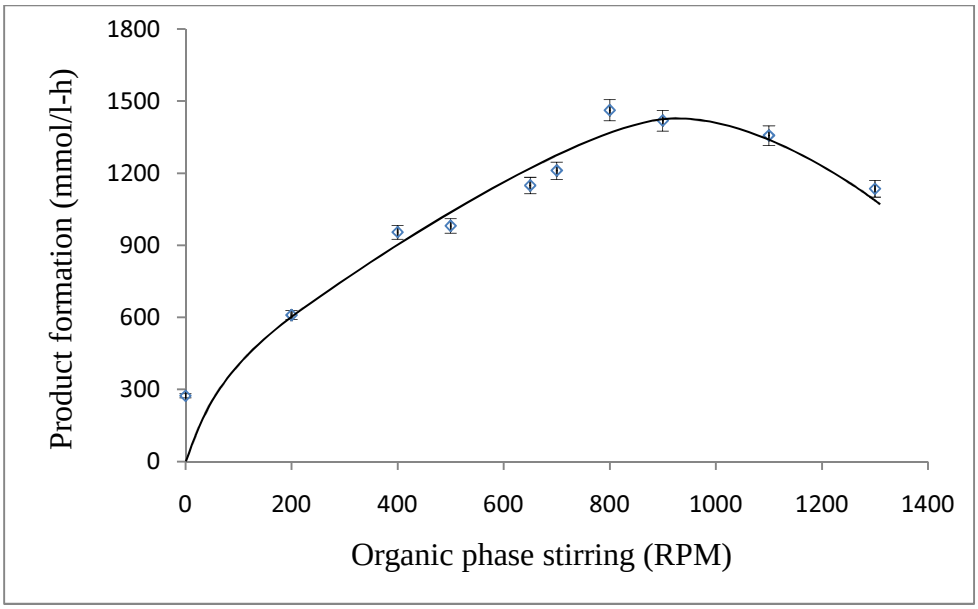

Fig 4.14: Effect of organic phase stirring on fatty acids production by immobilized lipase (enzyme amount: 5.14 g/m2), transmembrane pressure: 0.8bar; aqueous phase flow rate: 0.06m/s; temperature: 35 ˚C; pH -7 pure olive oil as organic phase).

4.4.4. Influence of axial velocity of the aqueous phase

Reactor performance is also affected by removal capacity of the reaction product from the enzyme-loaded membrane sites. The axial velocity of the aqueous phase has been studied in order to obtain the optimum axial velocity able to promote fatty acids removal from reaction site. Six different modules has been studied in four different axial velocity (**Table 4.5**). The standard deviation and the percentage of error has been calculated which is around 3%. In **Fig 4.15** each point represents the average of all the experiments done with six different modules.

Table 4.5: Study of different module in different axial velocity

	Axial Velocity (m/s)			
Exp.No	**0.04**	**0.06**	**0.08**	**0.09**
Module 19	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3
Module 20	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3
Module 21	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3
Module 22	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	-----	-----
Module 23	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3
Module 24	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3

With the increasing of axial velocity the mass transfer coefficient increases, which should result in an increase of extraction rate of the reaction products soluble in the aqueous phase. Results illustrated in Fig. 4.15 show that the reactor performance is almost constant as a function of aqueous phase axial velocity. It is worth mentioning that, the range in which the axial velocity of aqueous phase can be increased is limited by the corresponding increase of back pressure from lumen to shell (as the aqueous phase is recirculating along the lumen side of hollow fibers).

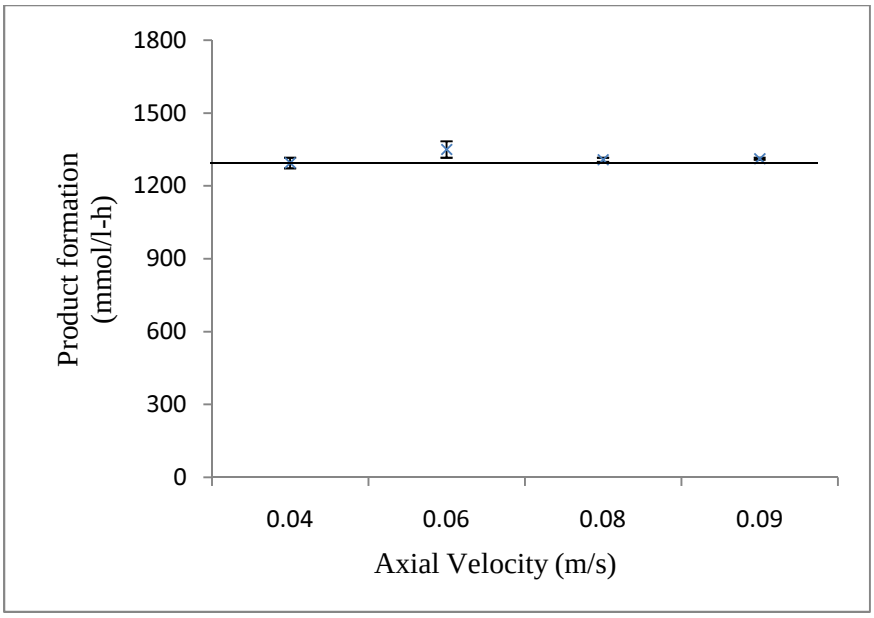

Fig 4.15: Effect of different aqueous flow rate on fatty acids production by immobilized lipase, (enzyme amount: 5.14 g/m^2), transmembrane pressure: 0.8 bar, organic phase stirring: 850 rpm; temperature: 35 ˚C; pH -7, pure olive oil as organic phase).

For higher values out of the range reported in the graph, transmembrane pressure from shell to lumen was not constant and water droplets in the organic phase could be observed.

4.4.5 Influence of TMP

Five different transmembrane pressure, from 0.6 bar to 1.0 bar at 0.1bar interval, have been investigated. The several modules used are summarized in **Table 4.6**. The standard deviation and the percentage of error has been calculated which is around 3%.

Table 4.6: Study of different module in different TMP conditions

	Transmembrane Pressure (bar)				
Module .No	**0.6**	**0.7**	**0.8**	**0.9**	**1.0**
Module 25	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3
Module 26	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3
Module 27	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3
Module 28	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	-----	------	--------
Module 29	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3
Module 30	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3	Exp#1 Exp#2 Exp#3

Fatty acid production with time at different transmembrane pressures is shown in **Fig. 4.16**. It can be seen that the reactor performance increases with increasing the TMP up to a certain range.

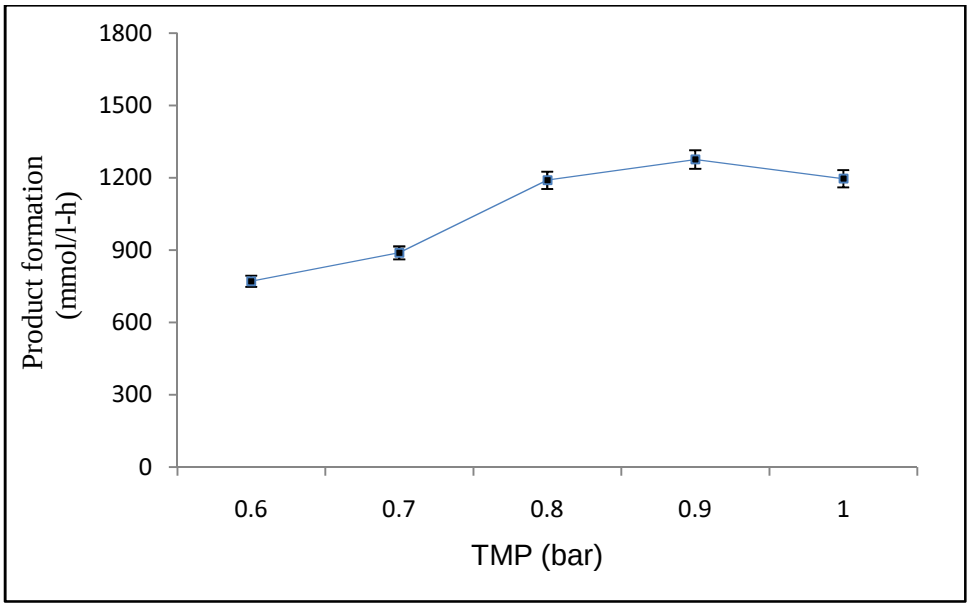

Fig 4.16: Effect of TMP on fatty acids production by immobilized lipase (enzyme amount: 5.14 g/m^2), aqueous phase flow rate: 0.06m/s; organic phase stirring: 850 rpm; temperature: 35 ˚C;pH-7, pure olive oil as organic phase).

These results could be explained as that increasing TMP in organic phase caused the higher penetration of the organic phase into the membrane thickness. So that moving the oil-water interfase more internally into the membrane and reducing the diffusion distances. As it is shown, after 0.8 bar the activity resulted constant up to 1 bar. Higher values have not been tested due to the effects on membrane sponge layer compaction at higher pressure. Therefore, a TMP value of 0.8 was chosen for subsequent developments using these membranes.

4.5 Test of the system with fried oil

After optimizing all the initial operating parameters we transfer the results to carry out the reaction with used cooking oil in order to verify its efficiency with

real streams to be processed. The fatty acid production as a function of time is reported in **Fig. 4.17**.

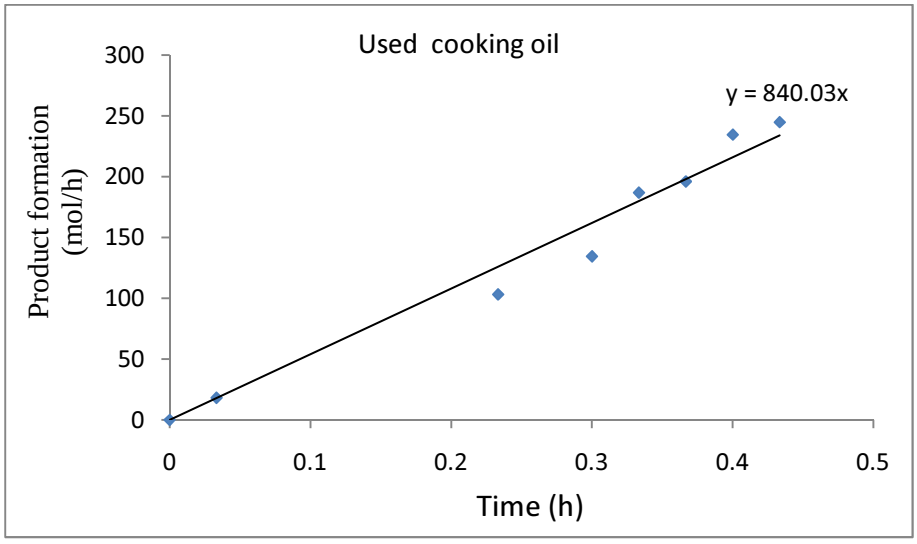

Fig 4.17: Fatty acids production by immobilized lipase with amount of enzyme: 5.14g/m^2 using fried oil : trans membrane pressure: 0.8bar; aqueous phase flow rate: 0.06m/s; temperature: 35 ˚C; pH -7,and organic stirring at 850 rpm

As it can be seen, the reaction product increases as a function of time, indicating that the enzyme-loaded membrane is able to work efficiently also with the waste oils. As glycerides content (in terms of both concentration and types, i.e tri- di- monoglycerides) in fried vegetable oils is different compared to the virgin olive oil a comparison of the reaction efficiency with the two substrate sources is not thorough. Nevertheless, an overall rough estimation of the effect of the raw material source on the reactor efficiency in terms of productivity (mol/h) has been attempted. It has been observed that the productivity of the reactor using fried cooking vegetable oils is 82 (± 2) % compared to the one obtained with virgin olive oil. The fact that the lower productivity was due to the oil composition could be confirmed by the fact that using again the virgin olive oil, the reactor performance was the one originally obtained with this pure substrate source, i.e. enzyme catalytic activity was not damaged by the waste fried cooking oils raw material.

Fig. 4.18 illustrates the behavior of the submerged biocatalytic membrane as a function of different types of fried vegetable oils. Three different measurements have been done with each of the three different type of fried oil and the standard deviation has been calculated. An error of about 8% was obtained.

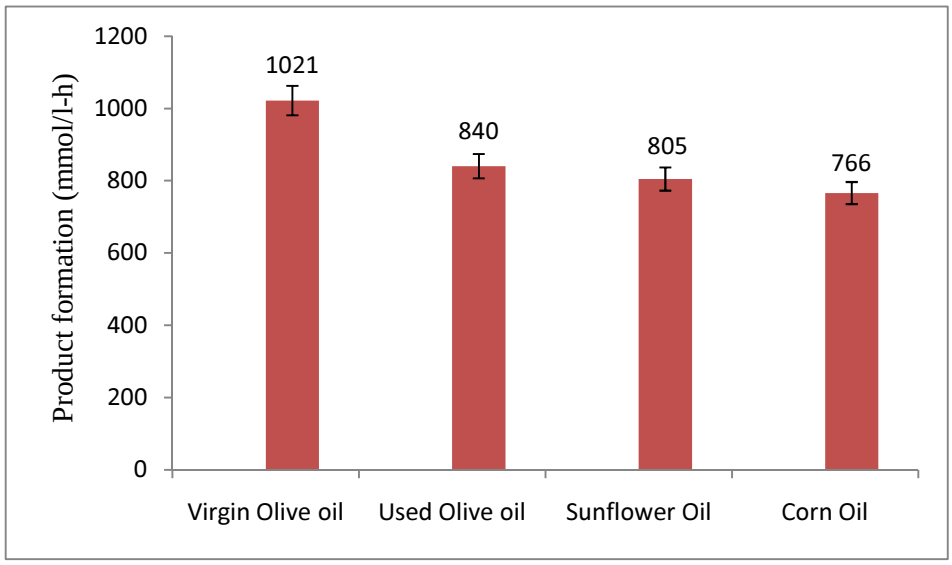

Fig 4.18: Behaviour of the submerged biocatalytic membrane with different types of fried vegetable oils. With amount of immobilized lipase: 5.14 g/m^2 using fried oil : trans membrane pressure: 0.8bar; aqueous phase flow rate: 0.06m/s; temperature: 35 ˚C; pH - 7,and organic stirring at 850 rpm

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Conclusions

In this work, a submerged biocatalytic membrane reactor with lipase-loaded membrane for the valorization of components present in waste oils has been developed. An experimental optimization of operating parameters has been carried. The aim of this study was to investigate the optimum reaction conditions of lipase from *Candida rugosa* on a hydrophilic support and investigate the effect of different substrates (waste cooking oil) on the hydrolysis reaction. The submerged enzyme-loaded membrane showed best performance at pH range between 6.5 and 7.5; temperature of 35 ˚C , transmembrane pressure of 0.8 bar, aqueous phase axial velocity of 0.06 m/s, organic stirring rate of 850 rpm. Virgin olive oil has been used for the optimization studies and then results have been applied for the hydrolysis of glycerides present in fried oils. Results confirmed that the SBMR can work with waste oils producing and simultaneously separating glycerides hydrolysis reaction products. In fact, the SBMR worked with an efficiency of about 82% when using fried olive oil compared to the use of virgin olive oil.

<u>The advances promoted in the present work include:</u>

1) Development of biocatalytic membranes with enzyme immobilised in submerged conditions which can be use for different applications including food, pharmaceuticals, biofuels.
2) Optimization the operating parameters of submerged biocatalytic membrane reactor for recovery of value added components.
3) Proof of principles of SBMR suitability for reaction with waste cooking oil.

Appendix

- Awards from 2008 to 2010

1. **Travel grant** for attending NAPOLYNET meeting at **Athens, Greece** in last 13th -15th May,2009.
2. **Full grant** to attend the EMS Summer School at **GKSS Research Centre in Geesthacht, Germany** from 29 Sept-2 Oct, 2009.
3. Awarded Travel Grant of AOCS to participate in 2nd International Congress on Biodiesel:The Science and Technologies to be held in **Munich, Germany** in November,2009
4. **Full grant** for attending Nanomemcourse on Nano-structured materials and Membranes for Health and Sustainable Water at Enschede (The Netherlands), from 7 Apr 2010 to 16 Apr 2010
5. **Grant** to attend the Advanced course on Food Application of Nanostructure Materials, Nanomemcourse, Cetraro and Rende ,Italy, 15 Sep 2010 to 24 Sep 2010

Relevant publication

S.Chakraborty, E.Drioli, L.Giorno, Development of a two separate phase submerged biocatalytic membrane reactor , Biochemical Engineering Journal,, (Submitted)

Book Chapter

Rosalinda Mazzei, Sudip Chakraborty, Enrico Drioli,, Lidietta Giorno, **Membrane Bioreactors in Functional Food Ingredients Production**, Membrane Technology, Volume 3: Membranes for Food Applications. Edited by Klaus-Viktor Peinemann, Suzana Pereira Nunes, and Lidietta Giorno ,Copyright © 2010 ,WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim ,ISBN: 978-3-527-31482-9

Proceedings

1. L. Giorno,S. Chakraborty, E. Drioli, "Membrane Bioreactors in Integrated Membrane Processes for Innovative Production Systems", NaPolyNet - Demonstration (Training) Course "Characterization Methodology & Tools for New Polymer Nanostructured Materials,Athens,Greece,13^{th} -15^{th} May,2009.

2. S. Chakraborty , L. Giorno, E. Drioli, Study on Enzymatic Immobilization in Submerged Membrane Bioreactor, XXVI EMS Summer School-Membranes for C02, , GKSS,Geesthacht ,Germany,29 Sep 2009-2 Oct 2009.

3. 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

4. S. Chakraborty , L. Giorno , E. Drioli , "Submerged Membrane Bioreactor for Recovery of High Value Component", IMeTI Workshop on "Membrane

Applications in Agrofood" 18 – 20 October 2009, Cetraro (CS) – Italy

5. S.Chakraborty , L. Giorno, E. Drioli , "Hydrolysis of Olive Oil in Submerged Membrane Bioreactor for Innovative Production Systems:" 2nd International Congress on Biodiesel : The Science and Technologies, Munich,Germany, 15-17 Nov 2009

6. S. Chakraborty , L. Giorno, E. Drioli, "Enzymatic Hydrolysis in Submerged Membrane Bioreactor for Innovative Production Systems" The Indian Chemical Engineering Congress-2009 (CHEMCON-09), Visakhapatnam, Dec 27-30, 2009

7. S. Chakraborty , L .Giorno, E. Drioli, "Development of Submerged Membrane Bioreactor for Innovative Biofuel Production Systems", 1st International Conference on "New Frontiers in Biofuels", 18-19 Jan, 2010, New Delhi, India.

8. S.Chakraborty , L.Giorno, E.Drioli , Nano-structured materials and Membranes for Health and Sustainable Water, Nanomemcourse Enschede (The Netherlands), from 7 Apr 2010 to 16 Apr 2010

9. S.Chakraborty , L.Giorno, E.Drioli, Advanced course on Food Application of Nanostructure Materials, Nanomemcourse, Cetraro and Rende ,Italy, 15 Sep 2010 to 24 Sep 2010

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 June-3 July 2009, Prof. Heiner Strathmann.

2009- Participation to IMeTi Workshop on "Membrane Applications in Agrofood" 18-20 Ottobre, Cetraro, Italia.

Oral presentation in International congress 2008-2010

1. 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

2. *S. Chakraborty , L .Giorno, E. Drioli*, "Development of Submerged Membrane Bioreactor for Innovative Bofuel Production Systems", 1st International Conference on "New Frontiers in Biofuels", 18-19 Jan, 2009, New Delhi, India.