The significance of Submerged Ceramic Membrane systems for production oriented Bioprocesses

Dissertation
zur Erlangung des Grades
des Doktors der Ingenieurwissenschaften
der Naturwissenschaftlich-Technischen Fakultät III
Chemie, Pharmazie und Werkstoffwissenschaften
der Universität des Saarlandes

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Saarbrücken

2007

Tag des Kolloquiums : 04.10.2007

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Abstract

The objective of the investigations performed and compiled in this study was to develop submerged membrane systems, using microporous ceramic membranes for the high cell density bioprocesses developed for different products including proteins, metabolites, surfactants and polysaccharides. Characterization and selection of membrane suitable to specific purpose and development of module to be integrated within the bioreactor is discussed. The tests include biomass retention and product removal from yeast (chapter 4) and bacterial (chapter 5) fermentation. Furthermore, the problem of biofouling is discussed and methods for its control have been investigated. Next, the submerged membrane system is tested for controlled substrate feeding in aqueous-two-phase systems (chapter 6), which are usually limited in their use due to very low mass transfer rates between the two immiscible phases. To add on to the range of application of ceramic membranes, especially in submerged form, hydrophobization of the membrane is investigated (chapter 7). The idea is to integrate the bioprocess and the down stream processing via submerged membrane system, thus obtaining a sterile and continuous operation. Ceramic membranes have advantages over their polymeric counterpart owing to their mechanical and thermal strength and environmental friendliness.

Kurzfassung

Das Ziel der hier vorgestellten Forschungsarbeit war es untergetauchte Membransysteme zu entwickeln, unter der Verwendung keramischer Membranen mit Mikroporen. Diese Membranen wurden für Prozesse mit verschiedenen Produkten (Proteine, Metabolite, oberflächenaktive Substanzen und Polysaccharide) entwickelt. Die Auswahl und Charakterisierung der für den speziellen Zweck geeigneten Membran und die Entwicklung eines Moduls für den Bioreaktor wird diskutiert. Die durchgeführten Testungen beinhalteten Biomassenretention und Produktentfernung aus Hefen (Kapitel 4) und bakterielle Fermentation (Kapitel 5). Desweiteren wird das Problem des Biofouling diskutiert und Methoden erläutert, um dieses unter Kontrolle zu halten. Das Membransystem wurde hinsichtlich kontrollierter Substratzuführung getestet. Dies geschah in einem wässrigen Zweiphasensystem (Kapitel 6), welches

normalerweise aufgrund der niedrigen Massentransferraten zwischen den zwei Phasen in ihrem Nutzen sehr beschränkt ist. Um weitere Anwendungsmöglichkeiten der getauchten keramischen Membranen zu entwickeln, wurde die Hydrophobisierung des Membrans erforscht (Kapitel 7). Die Absicht dahinter war es den Bioprozess und den Downstreamprozess durch das getauchte Membransystem zu vereinen, um einen sterilen und kontinuierlichen Prozess zu erhalten. Keramische Membranen sind aufgrund ihrer mechanischen und thermischen Belastbarkeit und ihrer Umweltverträglichkeit den Polymermembranen gegenüber im Vorteil.

“failure is only the opportunity more intelligently to begin again”

- Henry Ford

to my family

Acknowledgments

I am extremely grateful to Prof. Horst Chmiel to provide me this opportunity to work at upt GmbH in Saarbruecken, Germany and pursuing my Ph.D. studies at Department of Process Technology, University of Saarland, Saarbruecken, Germany. He supervised me not only in various projects, which I did as a coworker at upt, but also guided me to work out this thesis out of various related but discrete projects. The professionalism and commitment to work that I have learned from him will go a long way in to my career.

I owe my sincere thanks to Prof. Christoph Syldatk to review my thesis and for his valuable suggestions during the project work in Karlsruhe, Germany.

I owe my deep sense of gratitude to Prof. Valko Mavrov for his encouragement and counseling with care and affection throughout my stay. Though he was not directly involved in any of the projects that are compiled in this thesis, but it was his moral support which kept me motivated to stay and finish the work in spite of the difficult situation that we faced at upt.

I would like to thank: the research group of Prof. Syldatk in Karlsruhe, Germany; the research group of Prof. Antranikian in Hamburg-Harburg, Germany; the research group of Prof. Heinzle in Saarbruecken, Germany; the research group of Prof. Manresa in Barcelona, Spain and the people at Pombiotech GmbH, Saarbruecken, Germany for their cooperation in the joint research work in the last three years that has been compiled in this thesis.

I express my heartfelt thanks to all my colleagues at upt, those who have been related to the projects I have been involved in and otherwise. Their friendly attitude and cooperation, in spite of my limited Deutsch, have been great throughout my stay here. The team spirit I have learned with them is invaluable. I owe them a huge share of my success that I attained here, as well as which I will achieve in future.

Thanks to Iris and Paula for being a great colleague, friend and family. A million thanks to my sister Neeta for the remarkable supply of literature. Especial thanks to Patricia and Sikander for their help in correcting the manuscript. Finally, I thank all my multinational-multicultural friends in Europe, especially in Saarbruecken for just everything they did to make my stay here an experience for a lifetime. I not only got to learn different cultures and professions but also gained a significant development in my outlook towards life in general.

- Anuj Dhariwal

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1 Introduction

For any bioprocess to be economically feasible, it is important to achieve high volumetric productivity. To this end, high cell densities are a prerequisite. Moreover, it is crucial to remove the product and the metabolic byproducts continuously from the reaction mixture in order to avoid their accumulation to the growth inhibitory level, and also to avoid any degradation of the product of interest [Daubert et al., 2003]. Downstream processing (DSP) is a vital step, which accounts for not only the total process-time and most of the process costs but also for the quality of the product and the overall practical feasibility of the bioprocess [Chmiel, 2006]. Thus, purification strategies must be planned carefully and the techniques used in the laboratory (bench scale) should consider future scale-up and feasibility of methods for production purposes. Effective integration of the different parts of the production process will provide tools for increasing the productivity and/or product yield and quality [Asenjo and Leser, 1996]. Integration of fermentation and a primary separation-step can accelerate the product formation, improve the product yield and facilitate downstream processing. A significant number of *in situ* product removal (ISPR) methods have been developed in the last two decades with the aim to improve productivity, decrease the total process time and make the process eco-friendly and cost-effective [Stark and Stockar, 2003].

The first step in any downstream processing chain is the separation of biomass from the rest of the medium, which contains the product, the byproducts and the unused substrate (if any). Membrane systems have found wide range of applications in this respect, especially as high biomass density is required in the bioreactor. The integration of membranes in the bioreactor has provided a logical attempt to gather in a single operation: bioconversion, product recovery, and/or concentration and biocatalyst recovery, a goal that has been successfully achieved and has found a wide range of applications. Membranes have proven to be efficient tools for biocatalyst retention and have been widely used in industrial processes [Yang et al., 2006]. The most common methodology is to use an external cross-flow membrane unit where the product is continuously separated from the system with permeate and

the biomass is recycled back to the bioreactor. However, these types of external systems not only demand extra reaction volume and high energy for maintaining continuous flow rates (and hence sufficient transmembrane pressure for the filtration) through the membrane unit, but could also pose problems with respect to sterilized operation of the bioprocess [Chang et al., 1994]. This is where the submerged membrane systems hold advantage over the classical membrane bioreactors.

Major applications of Submerged Membrane Bioreactors (SMBRs) that have been published so far are in wastewater treatment. But their advantage with space requirement, sterilization within the bioreactor and less energy requirement for continuous operation [Oever, 2005] make them amiable to investigate with the fermentation processes. However, they must be either more economical or yield better productivity as compared to conventional methods. This may involve modification of the intrinsic properties of the membranes, as well as redesign of membrane modules and membrane processes. Thus, customization of the membranes to the specifications of the bioreactor (geometry and the available space) and the bioprocess (pH, temperature, total reaction volume, flux requirement, etc.) will be the first step towards this integrated approach. Moreover, the effect on microorganisms due to their direct interaction with the membrane system should also be taken into account during such investigations.

Apart from the accumulation and degradation of the product, one more limitation needs to be taken care in order to enhance the overall productivity of the fermentation process, and this limitation comprises of the optimum concentration of the substrate in the medium, especially if the substrate is not easily miscible with the aqueous fermentation phase. Aqueous two-phase systems offer a valuable biotechnological tool for biotransformation of apolar compounds. However, such systems are limited in high mass transfer rates due to low miscibility of the two phases. To circumvent this, it is required to improve the organic-aqueous interfacial area in the reaction system [Buehler et al., 2002]. In addition to biomass retention and product recovery, one more application with submerged membranes could be investigated for substrate feeding in aqueous two-phase fermentation systems by direct emulsification within the aqueous fermentation medium [Chmiel, 2004]. Owing

to the hydrophilic character of ceramic membranes, this could be used to feed hardly-miscible oily substrates in aqueous medium and to enhance oxygen solubility by homogenous dispersion of oxygen-vectors (PFCs, silicon oil, etc.).

For the integration of a membrane system into a bioreactor following points should be taken into account:

- Selection of the membrane with optimum pore size for the required filtration, thus retaining the cells inside the bioreactor while allowing the desired product to go with the permeate.
- Stability of the product, its sensitivity to shear and volume of product to be processed.
- Design of the membrane module according to the flux requirement of the filtration process and the available space inside the bioreactor.
- Integration of the membrane module in the bioreactor. Not only should the compatibility with the other components of the bioreactor as well as with the bioreactor operation be taken care of, but also the long term stability and adaptability to change in operation.

1.1 Aim of the thesis

The aim of this work is to account for:

- the choice of membrane material compatible to the biochemical aspects of the bioprocess,
- characterization of the commercially available membranes for their suitability to a specific need and
- preparation of membrane modules and optimization of the operation with specific case-to-case requirements.

Fouling of the membranes with various constituents of the fermentation broth is addressed and methods are investigated for its control. Efficiency of the process is accounted not only in terms of productivity enhancement and smooth operation, but also the cost-effectiveness and suitability to upscale. Experimental data from five different projects have been used to formulate the results for the above mentioned investigation themes.

2 Theoretical background

2.1 High cell density fermentation

Fermentation, by definition, means the use of carefully cultured microorganisms, animal cells, and plant cells to produce products that are useful to humans. By this definition, fermentation is as old as history. Indeed, the earliest known document includes a description of brewing. Today, fermentation covers a vast area where chemically or biologically defined species are produced. The product could be the microorganism itself (for food purpose, etc.), a primary or secondary metabolite produced by that microorganism during its growth cycle, or a foreign compound for which the microbe is playing a host. In the last century, antibiotics eclipsed organic chemicals as the principal products of biotechnology. They have been supplemented by amino acids, enzymes and other medically useful proteins. With grown insight into the genetics of microorganisms, it is more and more possible to produce specifically the product of interest. A great deal of research work has been done and is still continuing, for the development of bio-processes which have smaller footprints and are easy to control, robust, environmentally friendly and cost-effective [Stanbury et al., 1999].

In general, when a microorganism produces a compound, the main criterion involved in the evaluation of process performance, according to economic consideration, is the volumetric productivity **P**, defined as:

$$\mathbf{P = \nu_p . X}$$

ν_p is the specific activity of the microorganism and **X** is the biomass concentration. Higher productivity can be obtained by increasing cell concentration provided that the specific activities are maintained [Daubert et al., 2003].

For high-volume-low-value products, such as ethanol, high cell density and high volumetric yield are essential conditions for economical feasibility of the overall process. Moreover, for low-volume-high-value products, such as antibodies, high cell density significantly reduces capital investment and operation cost of the GMP production facilities. This reduction in cost is achieved due to reduction in size of the

fermentation equipment, upstream utilities such as purified water, purified steam, clean air supply and clean room environments. The size of downstream process units is also being reduced. Thus obtaining a high-density cultures and hence improving the volumetric productivity is a major objective for any bioprocess [Shiloach and Fass, 2005].

The main problems arising from High-Cell-Density-Cultures (HCDC) are solubility of solid and gaseous substrate in watery media, limitation and/or inhibition of substrates with respect to growth, instability and volatility of substrates and products, accumulation of products or metabolic byproducts to a growth inhibitory level, degradation of products, high evolution rates of CO_2 and heat, high oxygen demand as well as increasing viscosity of the medium [Gupta et al., 2002].

A simple batch reactor, owing to its ease of operation, is very useful at laboratory scales to optimize the media composition as well as to study the behavior of cells under different growth conditions. However, they can not be employed when high biomass density and high productivity are desired. Microbial HCDC are usually performed under feeding conditions where specific nutrients are limited, in particular carbon sources like glucose, glycerol, methanol and others [Riesenberg and Guthke, 1999].

Types of bioreactors commonly used for HCDC include: common stirred tank reactors (STR) with the usual instrumentation for substrate feeding, STR with various types of external and internal cell retention, dialysis-membrane reactors with continuous removal of inhibitory or toxic compounds without additional stress to cells, gas-lift fermenters designed to improve the oxygen transfer rate, two compartments reactor system consisting of a STR and an aerated plug-flow reactor (PFR), etc. [Junker, 2004]. These growth strategies, together with optimization of media composition and the application of molecular biology methods, made it possible to grow various microbes to very high cell densities. Common examples include *E. coli* being grown up to 190 g/l dry cell weight and *P. pastoris* up to 450 g/l wet cell weight [Shiloach and Fass, 2005]. All the bioreactor types are very valuable for research purposes. However, for high cell density fermentations in industry, the simple STR reactor under fed-batch operation is the reactor of choice because of its simplicity, its

potential for high productivity, its suitability for robust fermentation and above all its wide distribution [Riesenberg and Guthke, 1999]. A high-density culture can be achieved either by immobilization of the biocatalyst in the reactor or by retaining the cells in the fermenter via cell recycling or cell retention using membrane filtration.

2.2 Downstream processing (DSP)

Fermentation technology is commonly divided into 'upstream' and 'downstream' processing, which is primarily a way of saying bioreaction and bioseparation [Lightfoot and Moscariello, 2004]. The efficient separation and concentration of fermentation products plays a key role in the commercial success of a process. Even if the fermentation is successful, recovery of the product can be a major bottleneck [Patnaik, 1995]. It is well known, especially in the pharmaceutical industry that down streaming is the most expensive and unfortunately the most ineffective part of a bioprocess. Hence, a lot of research is devoted to development of new and more effective procedures to separate products from the reaction mixture [Stark and Stockar, 2003]. In general, the Downstream processing (DSP) has, in most of the cases, four steps that occur sequentially as:

1) Removal of insoluble: filtration and centrifugation are the principal unit operations used in this segment. Relatively little product concentration or improvement of product quality occurs.
2) Isolation of the product: the steps, which are relatively nonspecific, remove materials of divergent properties compared to the desired product. Appreciable concentration and product-quality increase usually occur. Adsorption and solvent extraction are typical.
3) Purification: these processing techniques are highly selective for the product and remove impurities of similar chemical functionality and physical properties. The main examples are chromatography, electrophoresis and precipitation.
4) Polishing: the end use of the product dictates the final sequence utilized. Crystallization is often the key. Most of the products must also be dried.

All these steps have been described in detail with relevant examples from the last 20 years by Chmiel [2006], and are schematically shown in figure 2.1.

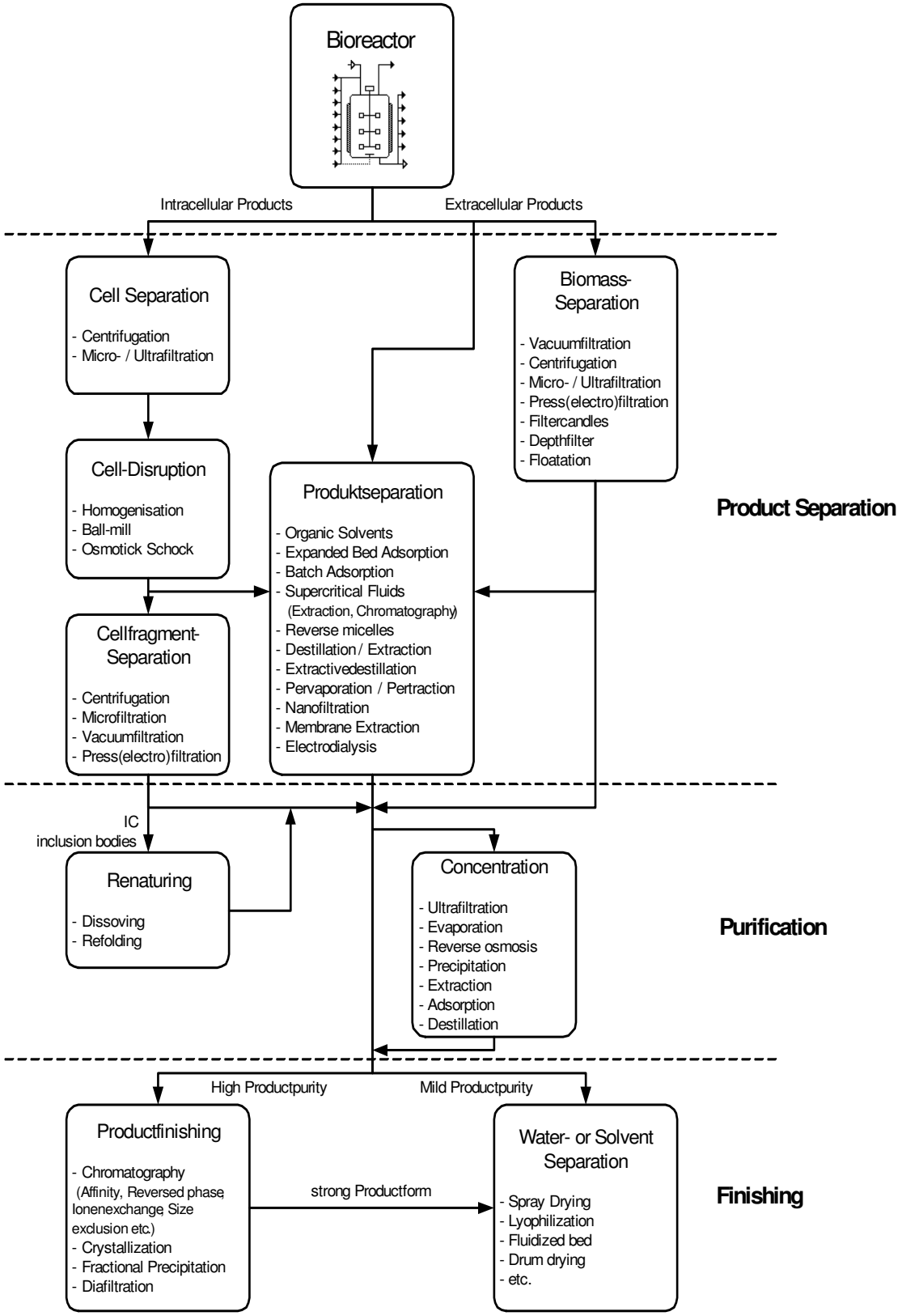

Fig. 2.1: Schematic summary of downstream processing (DSP).

2.3 *In situ* product removal (ISPR)

2.3.1 Integrated bioprocess

The efficient separation and concentration of fermentation products play a key role in the commercial success of a process. Thus, purification strategies must be planned carefully and the techniques used at the laboratory and bench scale must consider future scale-up and feasibility of methods for production. For this, effective integration of the different parts of production will provide tools for increasing the productivity and/or product yield and quality [Asenjo and Leser, 1996].

The concept of process integration is related to following considerations:

- Reduction of the number of unit operations: rational reduction of the number of unit operations improves the process in the sense of economics and compactness. This may be performed by cutting out an operation. By the use of combined (integrated) unit operations and by a combination of former possibilities.
- Reduction of process streams: full conversion within a single, integrated unit operation instead of voluminous recycle flows reduces process streams in general and thereby energy consumption and waste production. Selective removal of products also decreases the water usage in general and the effluent of waste water in particular.
- Control aspect: in integrated unit operations, the rate of product formation and the rate of product withdrawal from the reactor are decoupled, adding an extra degree of freedom which improves, in general, the control over the process. In the case of biomass retention in the fermenter, by means of membrane or centrifugal techniques, the productivity of the bioreactor will improve substantially because high concentrations of the biocatalyst are achieved.

Several biotechnological processes are impaired by product inhibition or by formation of toxic byproducts during cultivation. Sometimes product decomposition during its formation diminishes the productivity and product yield. To avoid these drawbacks, the product is removed from the reactor during its formation by a procedure known as '*in situ* product removal' (ISPR). This approach includes operational steps such as

separation of cells from process liquor, product extraction followed by reintegration of cells and supernatant into the cultivation [Schuegerl and Hubbuch, 2005].

2.3.2 Application of ISPR in biotechnology

In situ product removal can increase the productivity or yield of a given process by any of the following means: 1) overcoming inhibitory or toxic effects of product to allow continuous formation at maximal production level, 2) minimizing product losses owing to degradation or uncontrolled release, 3) shifting unfavorable reaction equilibrium and 4) reducing the total number of downstream processing steps. However, ISPR is restricted to extracellular products, because of the high difficulty of releasing intracellular products without affecting cell viability. Furthermore, ISPR is also applied to remove by-products that lower the performance of a fermentation process [Lye and Woodley, 1999].

Selection of an ISPR technique for the removal of a specific envisaged product from a complex medium depends mainly on the chemical and physical characteristics by which it differs from other medium components. In cases where these differences are distinctive, ISPR techniques based on product evaporation, extraction and size-selective permeation have been successfully applied, resulting in enhanced yield and productivity for a number of products [Dukler and Freeman, 1998].

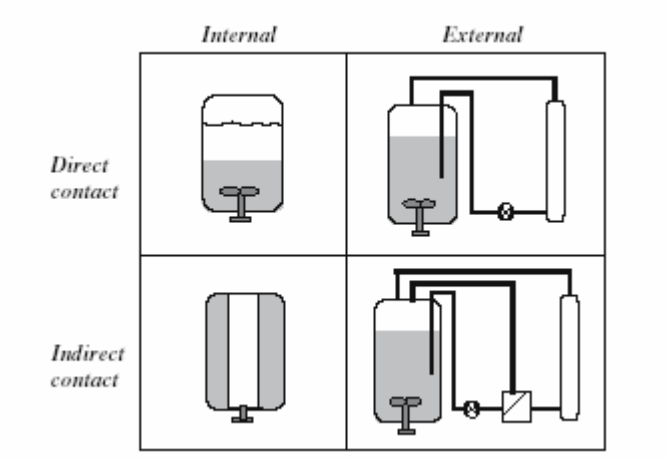

Fig. 2.2: Different configurations of ISPR [Buque-Taboada et al., 2004].

The success of an ISPR process does not depend only on the chosen separation technique but also on the configuration of the bioreactor/separation units (fig. 2.2) and mode of operation. Stability and robustness of a process is reduced if the cells

are in direct contact with the separation phase. Stable emulsions are formed if a water-immiscible solvent and a living cell-containing aqueous phase are mixed vigorously. But cells can form a biofilm on the adsorbent material, and thereby reducing the adsorption capacity of the particles [Stark and Stockar, 2003]. Membrane systems offer vital solution in these respects and are discussed in detail in the next sections.

2.4 Membrane applications in biotechnology

Membranes are an important part of industrial chemistry, for example, in unit operations such as distillation, extraction and filtration. Within the living cells, membrane processes are crucial for its survival and reproduction, and this is also true for higher level organisms. Hence, it is obvious that membranes and membrane processes play an important role in biotechnology — in the use of microorganisms and isolated enzymes and in the product recovery [Luetz et al., 2006]. Many industrial processes that rely on purification are switching to membrane filtration because of the versatility of the technology (table 2.1), and because membranes produce a consistent end product with less energy and at lower costs [Lamminen et al., 2006]. Coupled with biological treatment processes, membrane technology has gained considerable attention due to its wide range of applicability and the performance characteristics of membrane systems that have been established by various investigations in the last 15 years [Visvanathan et al., 2000].

Typically, fermentation broth contains many different classes of material including suspended solids, colloidal solids, microorganisms, cell debris, macromolecular solutes and microsolutes, including both primary and secondary metabolites. The particulate size of this material may range form 10 angstrom to several mm in diameter [Cook, 1996]. Membrane systems offer a wide range of filtration and are well categorized in table 2.1. Furthermore, application of membrane technology for the recovery of fermentation products has grown rapidly because a substantial part of the technological and financial success of bioprocess depends on the post-fermentation steps [Patnaik, 1995]. As already discussed in the previous chapter about down stream processing, the first step is always to obtain a cell free effluent which is then processed through various steps, depending on the nature of the

product. However, it is very important to integrate this first step in a continuous mode with rest of the DSP steps to enhance product recovery and to reduce process time and total process costs.

Table 2.1: Membrane applications in biotechnology [Chmiel, 2006].

Pressure-driven membrane processes

Membrane separation process	Separation principle	TMP (bar)	Pore diameter/ cut-off	Biotechnological applications
Microfiltration	Sieving effect	0.1 - 2	100 – 2000 nm	Removal of high molecular weight products from the bioreactor/ separation of suspended matter
Ultrafiltration	Sieving effect	1.0 – 5.0	5 – 100 nm 10^3 – 10^5 D cut off	Removal of low molecular weight products from the bioreactor/ concentration of macromolecular solutions
Nanofiltration	Sieving effect, solution diffusion and membrane charge	5.0 – 20.0	0.5 – 10 nm 10^2 – 10^3 D cut off	Separation of organic and inorganic components in the aqueous solution
Reverse osmosis	Solution/ diffusion	10.0 – 200.0	<100 D cut off salt retention 90%	Concentration of solution with very low molecular weight compounds

Membrane processes based on solution-diffusion principle

Membrane separation process	Driving force for mass transport	Separation principle	Biotechnological applications
Dialysis	Concentration difference	Symmetrical porous membranes	Separation of low molecular weight compounds with inhibitory properties
Pervaporation	Partial pressure difference	Different diffusion through the membrane	Separation of low volatile components
Gas-separation	Partial pressure difference	Different diffusion through the membrane	Separation of gas and steam mixtures
Pertraction	Partial pressure difference	Different diffusion through the membrane	Separation of liquid mixtures

Membrane systems take advantage of their selectivity, high surface-area-per-unit-volume and their potential for controlling the level of contact and/or mixing between the two phases. They are very well suited to the processing of biological molecules since they operate at relatively low temperatures, pressures, and involve no phase changes or chemical additives. Hence, the extent of denaturation, deactivation, and/or degradation of biological products is minimized [Charcosset, 2006]. There are four main areas in typical microbiological production processes where membranes

can successfully be used: 1) sterilization of bioreactor feed systems, 2) downstream processing of spent medium, 3) retention or immobilization of biocatalyst and 4) on-line monitoring of bioreactor constituents [Luetz et al., 2006].

2.4.1 Biocatalyst retention/immobilization

To enhance the productivity and hence overall commercial significance of any bioprocess, high cell densities inside the bioreactor are a prerequisite. This is achieved either by retaining the biocatalysts inside the reactor loop (by recycling them back while drawing out the product with the permeate), or by immobilizing them in the reaction volume (fig. 2.3). Thus, the membrane acts not only as a selective barrier, but also as a carrier and catalyst. Membrane based cell recycle is most widely used in laboratory scale experiments. Unlike sedimentation and filtration, the membrane-based technique allows complete recycling of cells. In addition to improving productivity, the recycle culture has the benefit of increasing the average cell age in the reactor and this can be useful in production of secondary metabolites [Chang et al., 1994]. Depending on the case, the membrane could be a microfiltration (MF) or ultrafiltration (UF) membrane. Module configurations include hollow fiber, tubular, flat plate, spiral wound and rotating device. The two standard modes of operation are dead-end and cross-flow configurations [Charcosset, 2006]. The filtration step can be integrated with the rest of down stream processing in continuous or semi-continuous mode.

The other method is immobilizing the biocatalyst (whole-cell or enzyme) in the membrane matrix via adsorption or chemical bonding. The biocatalyst is thus unable to penetrate through the membrane, and the nutrients are fed under a concentration or pressure gradient into the substructure through the membrane, where they are converted by the catalyst in to the desired product. The product leaves the system through the membrane while the catalyst is retained [Strathmann, 1985]. Membrane systems have also been investigated for cofactor retention, which are then responsible for activation of their respective enzymes in the reaction mixture. The cofactor can be expended in size with the help of polymers to ease their retention on membranes and are regenerated chemically, electrochemically or photochemically. Whole-cell retention can found application in most unusual areas such as, exhaust air

purification. The disadvantages include lower biocatalyst activity, low specificity, mass transfer limitation and slow reaction rate.

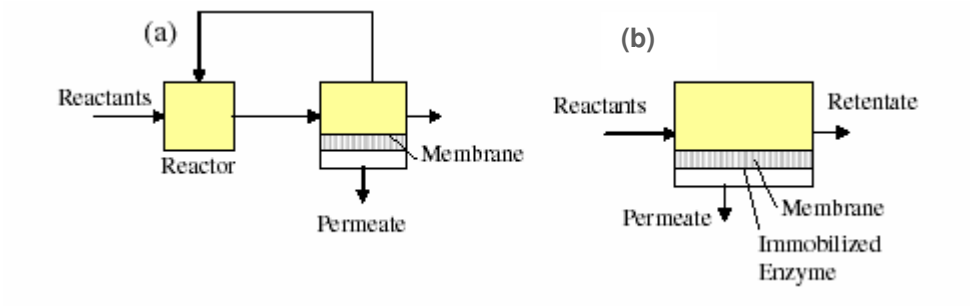

Fig. 2.3: Membrane bioreactor configurations: (a) reactor integrated with membrane unit for cell separation, (b) reactor with membrane active as a catalytic and separation unit [Charcosset, 2006].

2.4.2 Product recovery

To avoid growth inhibition due to product accumulation in the reaction volume, as well as to avoid degradation of the product due to unwanted secondary reactions or due to denaturation, it is crucial to separate the product from the reaction stream as soon as it is formed. Specific properties of membranes and membrane processes allow them to replace several, if not all, of the conventional separation steps. For example, highly volatile material such as acetone, ethanol and butanol, usually removed by distillation, can also be separated from the fermentation mix by pervaporation — a membrane process. Various factors why pervaporation is preferred over conventional methods for biofuel recovery from fermentation systems include increased energy efficiency, integration with the bioreactor, higher selectivity in continuous mode and synergy of performing both alcohol recovery and solvent dehydration [Vane, 2005].

Other known application of membranes for product separation method belongs to dialysis and electrodialysis. Classical recovery of pyruvate, one of the most important metabolites in central metabolism, is an energy intensive process that requires use of heavy metals. The electrodialysis with UF membrane has a high selectivity. It has been shown that this approach is well suited for separating pyruvate from fermentation broth, even as a fully integrated ISPR approach. Under optimized conditions, pyruvate could be produced at a concentration of 900 mmol L^{-1} [Zelic et al., 2004].

One of the main advantages of membrane systems for the initial recovery of antibiotics from a fermentation broth is the ability to obtain very high yield, using a combined filtration and diafiltration process. Ultrafiltration may also be used to remove emulsifiers in antibiotic broths before solvent extraction in order to avoid emulsification and to improve extraction efficiency [Li et al., 2004].

Furthermore, membrane separation systems can be used to increase the oxygen content in the air to 50% by selective removal of nitrogen [Chang et al., 1994]. Similarly, selective continuous removal of CO_2 from fermentation broth can be applied as a foam reduction technique in processes limited by excessive foam, like in surfactant production bioprocesses. Oxygen separation technology has applications in health care, defense, on-site generation of gases with known oxygen concentration, food packaging (oxygen removal), aquaculture, etc. [Ciacchi et al, 2002].

Much effort is still being devoted to develop new membrane modules with improved mass-transfer characteristics for ultrafiltration and microfiltration processes. This includes rotating disk filters, cylindrical taylor vortex devices, conical shaped rotors and helical coiled Dean vortex systems.

2.4.3 Substrate feeding

Microbiological production must be carried out under sterile condition. Although, there are many means of sterilization available, use of MF membranes is one of the most efficient and reliable methods for sterilization of gases and of liquids with low-molecular-weight components. The components are not subjected to the thermal stress or chemical alteration associated with heat treatment procedures, chlorination or ozonation. When used properly, sterile filtration is extremely reliable and can be carried out with a minimum cost [Strathmann, 1985].

Another new application with respect to feeding is bubble-free gassing into the fermentation medium. In the case of mammalian cells, the introduction of bubbles of oxygen or air can cause significant hydrodynamic stress, which could be harmful or even lethal to the cells. The enormous energy released when a bubble bursts can damage the cells. Bubble-free gassing via introduction of oxygen through a dense

silicon membrane can solve this problem. Efficient mixing of the gas in the liquid also leads to a low radial oxygen gradient. Similarly, bubble-free hydrogen gassing has been developed for regeneration of hydrogenase. The diffusion of hydrogen through the porous PTFE membrane occurred at high pressure, and the method has been described for the production of NADPH and for the production of biosensors and biofuel cells [Luetz et al., 2006].

If the substrate has a different solubility to the product, a biphasic membrane reactor can be used. In this type of system, the enzyme-loaded membrane is located between two immiscible liquid phases, an organic and an aqueous phase. The organic phase contains the substrate, which is flushed along one side of the membrane; the substrate is transported (by diffusion) to the enzyme, where the reaction takes place, and the product is extracted into the aqueous phase and flushed along the other side of the membrane. If the biocatalyst is selective for only one of the two enantiomers present in a racemic mixture, a biphasic system is particularly useful for producing pure enantiomers. Such systems have already been applied for: the hydrolysis of pectines in fruit juices, the treatment of wine, the treatment of milk and cheese whey, the treatment of oils and fats and various processes in biomedical and pharmaceuticals [Charcosset et al., 2004]. Application of membrane system for emulsified feeding of substrate in aqueous two-phase system is discussed in detail in chapter 6.

2.4.4 Membrane-moderated sensors

The online monitoring of bioreactor constituents is important in controlling and optimizing a biocatalytical production process. Conventional methods such as pH or O_2-measurement can be carried out on-line, but viscosity or density measurements are rather nonspecific. Membranes can be used for the development of specific analytical sensor. For example, a typical enzyme electrode consists of an asymmetric double-skinned microporous membrane in which an enzyme is immobilized. One side of the membrane faces the feed solution containing the component to be monitored; the other side is in contact with a reference solution containing an electrochemical analytical device, such as a potentiometric electrode [Strathmann, 1985]. Since

various enzymes are available which react specifically with certain components, quite a variety of specific membrane-moderated enzyme electrodes has been developed.

2.4.5 Other applications

Adsorptive membranes have been studied as an alternative to conventional resin-based chromatography columns. The benefits include shorter diffusion times, less operating pressure, high hydraulic fluxes and better mechanical strength. The interaction between molecules and active sites on the membrane occurs in convective through-pores, rather than in stagnant fluid inside the pores of an adsorbent particle. Single-use membrane chromatography cartridges with reduced labor and buffer requirements resulting in improved process economics [Reis and Zydney, 2001]. Overall, membrane applications in bioprocesses are schematically summarized in figure 2.4.

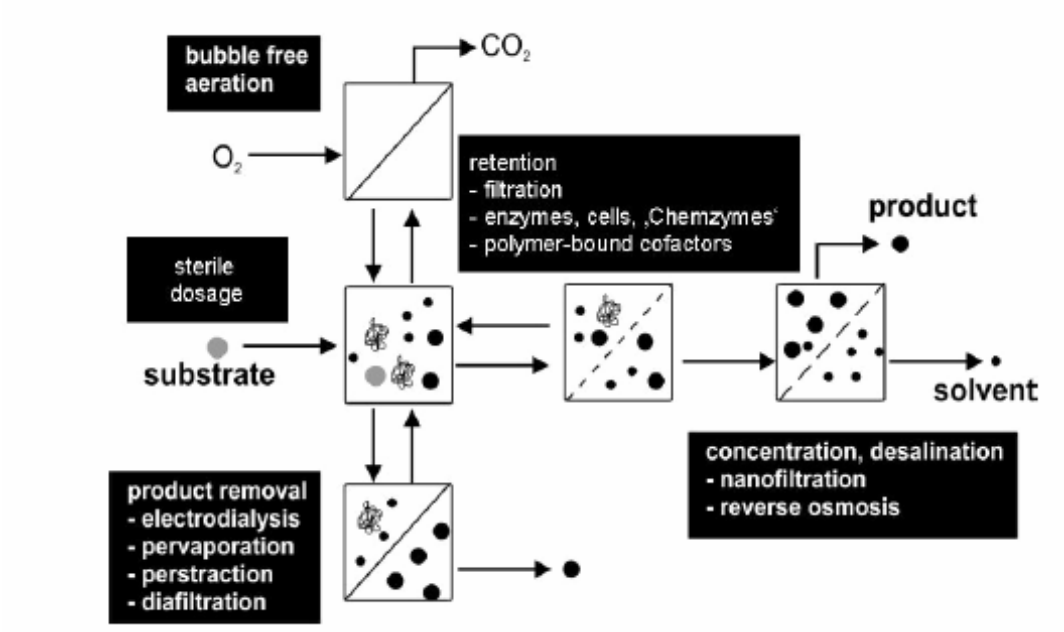

Fig. 2.4: Schematic summary of membrane applications in bioprocesses [Luetz et al., 2006].

2.5 Membrane bioreactors (MBRs)

Membrane bioreactors were developed around the concept of physically separating biocatalyst and substrate and/or products using a semi-permeable synthetic membrane. The biocatalyst is thus confined to a defined zone in the membrane reactor, while substrate and products flow across the membrane either by diffusion

(induced by concentration gradients) or by convection (generally induced by pressure gradients) [Reis and Zydney, 2001].

There are two modes of operation — constant transmembrane pressure (TMP) or constant flux. In a constant TMP operation, deposition and fouling cause a decline in flux that is initially rapid but becomes more gradual. For constant flux, the effect of deposition and fouling is to increase TMP that is initially gradual but accelerates prior to cleaning. Constant flux is the preferred mode of operation for membrane bioreactors because it ensures a steady throughput [Fane and Chang, 2002]. With respect to productivity, the best operating conditions for a fermentation process are high product concentration and dilution rates [Daubert et al., 2003].

Membrane reactors were classically grouped according to the hydrodynamic/configuration of the system in CSTR and PFR types. However, these types proved unable to compromise some commonly used types in UF, such as flat membranes or dead-end operated modules and multiphase bioreactors. A classification based on the contact mechanisms that bring together substrate and biocatalyst was thus proposed. Therefore, membrane reactors could be divided into direct contact, diffusion contact, and interfacial contact reactors and are described in detail by Fernandes et al. [2003].

2.5.1 Why submerged membranes: SMBR?

Membrane based external cell recycle systems have been widely and successfully investigated for anaerobic systems, for production of ethanol, lactic acid and various other products resulting from anaerobic fermentation of bacteria or yeast. However, aerobic systems have not shown so much success because oxygen transfer limitation limits the advantage of high cell density operation, especially the lack of oxygen in the recycle loop. Other drawbacks of external cell recycle system are the costs of pumping and inhomogeneities in pH, dissolved oxygen and nutrient limitation while the cells stay in the external loop. Further, the sterilization of this device is difficult especially for long run continuous fermentation [Chang et al., 1994]. The answer to these limitations could be submerged-membrane bioreactor (fig. 2.5).

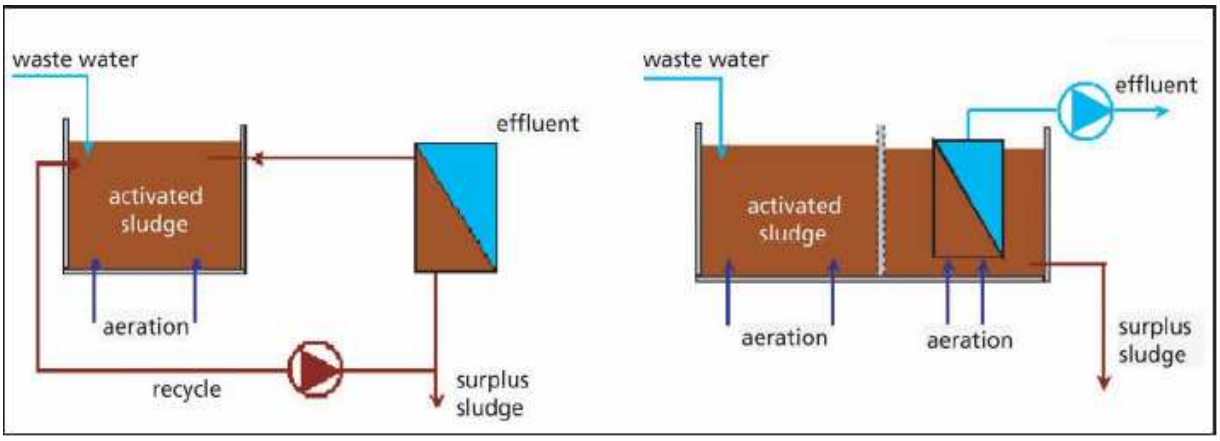

Fig. 2.5: Membrane bioreactor system; external cross-flow (left), submerged (right) [Oever, 2005].

External MBRs were considered to be more suitable for wastewater streams characterized by high temperature, high organic strength, extreme pH, high toxicity and low filterability. Treatment of municipal wastewater with MBRs mostly utilized the submerged configuration. Although studies on drinking water and groundwater treatment involved mostly external MBRs as pesticide removal and denitrification were usually part of the objective, there is no apparent reason why SMBRs could not be employed in this area. The energy and space saving characteristics of submerged membrane bioreactors have prompted many to view them as the most efficient and cost effective wastewater treatment technology [Oever, 2005]. The complete retention of sludge allows operation at much higher biomass concentrations. The higher the concentration the lower the F/M ratio becomes, with the effect that the microorganisms utilize a growing portion of the carbon content of the feed for maintenance purposes and less for growth [Rosenberger et al., 2002]. Moreover, in this technique the refreshing of feed along with the membrane is achieved by pneumatics (aeration) rather than hydraulics; a significant reduction in cost can be obtained if the membranes are cleaned by means of air scouring, rather than by cross-flowing of the feed solution [Goldsmith, 2004; 2005].

Several relatively large immersed membrane installations demonstrate the capability of the industry to successfully implement large and small submerged-membrane installations. Moreover, for a continuous bioprocess, sterile operation is a crucial issue and is greatly affected by the sterility of the integrated units. SMBRs offer a unique advantage in this respect as they are wholly integrated within the bioreactor.

Thus, no extra care needs to be taken and the whole system is sterilized at the same time.

The advantages of submerged membrane system over external cross-flow system could be summarized as:

- No need for fluid circulation:
- Low transmembrane pressure (TMP);
- Simple operation;
- High biomass density;
- Homogeneity in reactor (pH, DO, cell mass)
- Long term sterility.

However, there are some limitations which need to be taken care of:

- Decrease in flux due to fouling;
- Limited surface to volume ratio;
- Inflexibility [Yang et al., 2006].

Asymmetric membranes consist of a very dense top layer or skin with a thickness of 0.1 to 0.5 μm, supported by a thicker sublayer. The skin can be placed either on the outside or inside of the membrane and this layer eventually defines the characterization of membrane separation. Generally a submerged membrane should be outer skinned in general, permeate is extracted by suction, or less commonly, by pressurizing the bioreactor [Visvanathan et al., 2000].

The membrane could be a flat plate type, a tubular or a hollow fiber type. As hollow fiber elements are less expensive to produce than the plate-and-frame modules and are also backflushable, they are the preferred type with SMBRs. On the other hand, because the hydrodynamics are less readily controllable in such systems, they are more prone to fouling than either flat plate or tubular modules and therefore require more frequent washing and cleaning [Judd, 2004; 2005].

2.6 Ceramic Membranes

2.6.1 General features

The advantages of inorganic membranes have been recognized for a long time. Process stability, high availability, low requirement for preliminary treatment and minimum need for support and maintenance are the main features demanded by plant engineers and plant operators with respect to membrane installations. Ceramic membranes feature remarkable mechanical strength, which provides the operational advantage of not being subject to compaction as in the case of organic membranes under relatively high pressures [Hsieh et al., 1991]. Moreover, they can be easily sterilized by steam or by chemicals like active chlorine, owing to their high temperature and corrosion resistance [Matsumoto, 1988]. Hence, they offer advantage of being chemically inert and stable at high temperatures — conditions under which polymer membrane fails to offer long working life.

A major obstacle to establishing microfiltration in ecology is the relatively low filtration rates compared to competitive processes. Here, ceramic membranes (coated and uncoated) show much higher fluxes than non-porous membranes when operated at their ideal temperatures [Buxbaum, 1997]. Micro-organisms also present strong affinity to organic membranes, so that fouling due to bacteria is very critical during filtration with this type of membrane. In contrast, interactions between cells and ceramic membranes are negligible [Riesmeier et al., 1984]. These features make ceramic membranes particularly suitable for food, biotechnology and pharmaceutical applications, where along with continuous interaction with microorganisms membranes are subjected to repeated steam sterelization and cleaning with aggresive solutions. Due to their significantly longer service life and low-trouble operation, ceramic membranes – despite their higher price per m^2 – are in many cases more eficient than their organic counterparts.

2.6.2 The manufacturing method

These microporous membranes are made up of aluminum, titanium or silica oxides. Pore diameters in ceramic membrane for ultrafiltration and microfiltration range from 0.01 to 10 μm. They are generally made by a slip coating-sintering method. Other

techniques, particularly sol-gel methods, are used to produce membranes with pores from 10 to 100 Å. Sol-gel membranes are the subject of considerable research interest particularly for gas separation applications.

In the slip coating-sintering method a porous ceramic support tube is made by pouring a dispersion of a fine grain ceramic material and a binder into a mold and sintering at high temperatures. The pores between the particles that make up this support tube are large. One surface of the tube is then coated with a suspension of finer particles in a solution of a cellulosic polymer or poly (vinyl alcohol) which acts as a binder and a viscosity enhancer to hold the particles in suspension. This mixture is called a slip suspension; when dried and sintered at high temperatures a finely microporous surface layer remains. Most commercial micro and ultrafiltration ceramic membranes are made this way. In sol-gel method, the substrate to be coated is a microporous ceramic tube formed by the slip coating-sintering technique. The solution coated on this support is a colloidal or polymeric gel of an inorganic hydroxide. These solutions are prepared by controlled hydrolysis of metal salts or metal alkoxides to hydroxides. Depending on the starting material and the coating procedure, the sol-gel process can make a wide range of membrane.

2.6.3 Reported applications

Various applications have been found for ceramic membranes in microfiltration and ultrafiltration processes [Hasegawa et al., 1991]. The first industrial-scale application reported was the concentration of whey proteins in 1980 followed by milk protein standardization. The study has shown that even 1.8 μm membranes can not only reduce the bacteria counts by two orders of magnitude but also skim 98% of the fats. The proteins however remain in the filtered milk [Merin and Daufin, 1989].

Other successful applications in food and beverage industry include: the filtration of beer [Finnigan and Skudder, 2000] and fruit juices [Bolduan and Latz, 2000], the production of oil-in-water emulsion consisting of vegetable oil as dispersed phase and skim milk as the dispersion phase [Joscelyne and Traegardh, 1999] and biological treatment of waste water coming out from food industry [Bloecher et al. 2002]. In another study a ceramic membrane coupled to a bioreactor has been used in a dual role, combining the alternate functions of system aerator and filter for

remediating food industry process waste [Scott and Smith, 1997]. Use is reported for purification of alkaline cleaning solutions from the dairy industry, thus making it possible to reuse the cleaning solutions for an extended period before discharge thus saving energy, water and chemicals [Traegardh and Johansson, 1998].

Countless applications have been reported for clarification and purification of drinking water [Guibaud, 1989; Ericsson and Traegardh, 1997; Bottino et al., 2001]. As they can remove bacteria and clarify water in a single step, they can therefore reduce appreciably the classic sequences of water treatment processes [Agoudjil et al., 2005]. More applications come from treatment of industrial waste water [Bloecher et al., 2003]. Filtration of hot liquids by means of ceramic membranes is of critical importance because high temperatures yield specific permeate outputs. No cooling is necessary as would be the case if using polymer membranes [Bolduan and Latz, 2000]. These features make them amenable to several liquid-phase separation processes in petrochemical applications [Deschamps et al., 1989; Guizard et al., 1994; Higgins et al., 1994].

Ceramic membranes based on an alumina support have been successfully employed for separating binary H2 and N2 gas mixtures [Conesa et al., 1999]. Oxygen separation technology has applications in health care, defense, on-site generation of gases with known oxygen concentration, food packaging (oxygen removal), aquaculture, etc. Ceramic membranes based on $O2^-$ or $O2^-$/electronic conducting materials have the potential to serve this market. Pure $O2^-$ conducting ceramic membrane technology can be used not only for production of oxygen but also for oxygen removal in gas streams and enclosures as well as for oxygen level control to produce calibration gases [Ciacchi et al., 2002].

In biotechnology, a number of important applications of ceramic membranes are related to bioreactors for enzymatic and microbial conversion processes, where they are used both as separators and reactors or catalyst, owing to their unmatched biocompatibility. Use of ceramic membrane microfilter as an immobilized enzyme reactor has been investigated [Harrington et al., 1992] and the study suggested that a ceramic microfilter reactor can be a desirable alternative to a packed bed of porous particles, especially when an immobilized enzyme has high activity and a low

Michaelis constant. The enzyme could be covalently attached to a polymer layer previously adsorbed on a ceramic support, with no effect on its activity [Magnan et al., 2004]. A ceramic ultrafiltration membrane retained both the organic phase and the lipase that was solubilized in the aqueous phase. The enantioselective hydrolysis was carried out in repetitive batch mode and the enzyme consumption was greatly reduced [Liese et al., 2002].

Modifications such as placing Kenics static mixer inside the tubular membrane has been investigated, which led to flux improvement of about 45%, reduction of operation time by 25% and the energy saving of about 40% [Krstic et al., 2007]. For the whole cell biocatalysis, tubular ceramic membranes are already used for spore immobilization from *Phanerochaete chrysosporium* [Sheldon and Small, 2005]. A porous tubular ceramic membrane has been impregnated with a β-cyclodextrin polymer to obtain a chiral-selective membrane to obtain the ability to separate the enantiomers of the racemic pharmaceutical chlorthalidone [Krieg et al., 2000].

Having narrower pore size distributions compared to polymer membranes, ceramic membranes are attractive in a number of filtration applications related to fermentation broths and can be used for either upstream or downstream processing. For example, alumina and other ceramic membranes of various pore size and geometries have been successfully used for filtration of fermentation media [Riesmeier et al., 1984], recovery of polysaccharide from sugar fermentation broth [Guibaud, 1989], separation of yeast from the broth and the clarification of thin stillage [Cheryan, 1994], microfiltration of *E. coli* to increase the cell density inside the bioreactor [Li et al., 1996], penicillin G recovery from *Penicillium chrysogenum* broth [Adikane et al., 1999], extraction of bacterial alginate from batch fermentation broths of *Azotobacter vinelandii* [Saude et al., 2002], isolation of antibiotics from industrial fermentation broths of *S. clavuligerus* [Brites-Alves et al., 2002], filtration of suspensions of the polymorphic yeast *Kluyveromyces marxianus* [Foley et al., 2005], the recovery of volatile fatty acids from liquid organic sludge [Kim et al., 2005] and for continuous chitinase production by *Paenibacillus* [Kao et al., 2007].

Membranes with a more hydrophilic character generally offer better performance in the presence of antifoams, lessening the effect of these ubiquitous foulants. Studies

have shown that a regenerated cellulose membrane exhibited almost no flux loss under conditions which severely fouled a polysulfone membrane. Most ceramic membranes are hydrophilic and also seem to offer a benefit in their ability to maintain performance in the presence of antifoams, which is mostly the case with aerobic fermentation [Wang et al., 2001]. Ceramic membranes based on zirconia and alumina have been incorporated in many fermentation processes, not only in the conventional applications like purification and concentration of antibiotics, vitamins, amino-acids, organic acids, enzymes, biopolymers and biopeptides to name a few, but also for newer applications with human blood derivatives, vaccines, recombinant proteins, cells culture and monoclonal antibiotics [Cueille and Ferreira, 1989].

Many liquids in the applications discussed above have high viscosity and ceramic membranes can withstand the resultant high shear stress imposed by these liquids when they are pumped past the membrane surface. Higher permeate flux, longer production runs between cleaning cycles and shorter cleaning times have been some of the major reasons why ceramic membranes have the edge over organic polymeric membranes.

To summarize the development of membrane systems for bioprocess application, following points should be taken into account:

- Stability of the product;
- Shear sensitivity:
- Volume of product to be processed:
- Upstream and downstream operation steps.

Considering these factors, the steps in the design process would include selection of membrane, module design, integration with bioreactor and pilot testing.

3 Material and Methods

3.1 Membrane characterization

3.1.1 Estimation of pore-size distribution

Microfiltration membranes with various pore sizes, supplied by different manufacturers, were characterized and tested to establish their suitability for the investigations undertaken. In general the description from the manufacturer regarding the pore size is quoted as 'd_{50}' , that means around 50% of the total pores are having reported pore size while others could be bigger or narrower. As ceramic membranes, unlike their polymeric counterparts, have unsymmetrical structure, it is important to determine their pore-size distribution that is decisive in determining the retention properties of microfiltration membranes. Not only the thin layer at the membrane surface (inside or outside) but also the support layers and the channels are accounted for. Preferable is a pore size distribution as narrow as possible which is also equivalent to a sharp cut-off.

Pore size distribution is determined by the flow-pore method whereby pore diameters and their proportional distribution are defined by allowing a gas to pass through a dry membrane as well as through a membrane wetted with a test liquid. The so-called bubble-point of the membrane, which is equivalent to the largest membrane pore, is also determined by this method. In principal, this pore should be smaller than the cells to be retained to ensure total retention. A general result from the flow-pore test comes out as shown in figure 3.1. As shown in the figure, on Y-axis one can read the number of pores in percentage against the respective pore size on X-axis. It can be easily calculated that how many pores lie in region of interest for that particular application of the membrane. The bubble point can be read at far right, and is important to note that the total number of pores near the bubble point should not be significant. Otherwise, the cells to be retained can find an easy passage through the larger pores.

Another criterion for characterizing a membrane was the determination of pure water permeability. This value represents a membrane's hydraulic efficiency that should be

as high as possible. This was tested simply by measuring the flux of water through the membrane under continuous vacuum suction and was reported in terms of litres of water collected through a unit area of membrane and in a unit time under one bar pressure. The flux units are l/(m²·h·bar) .

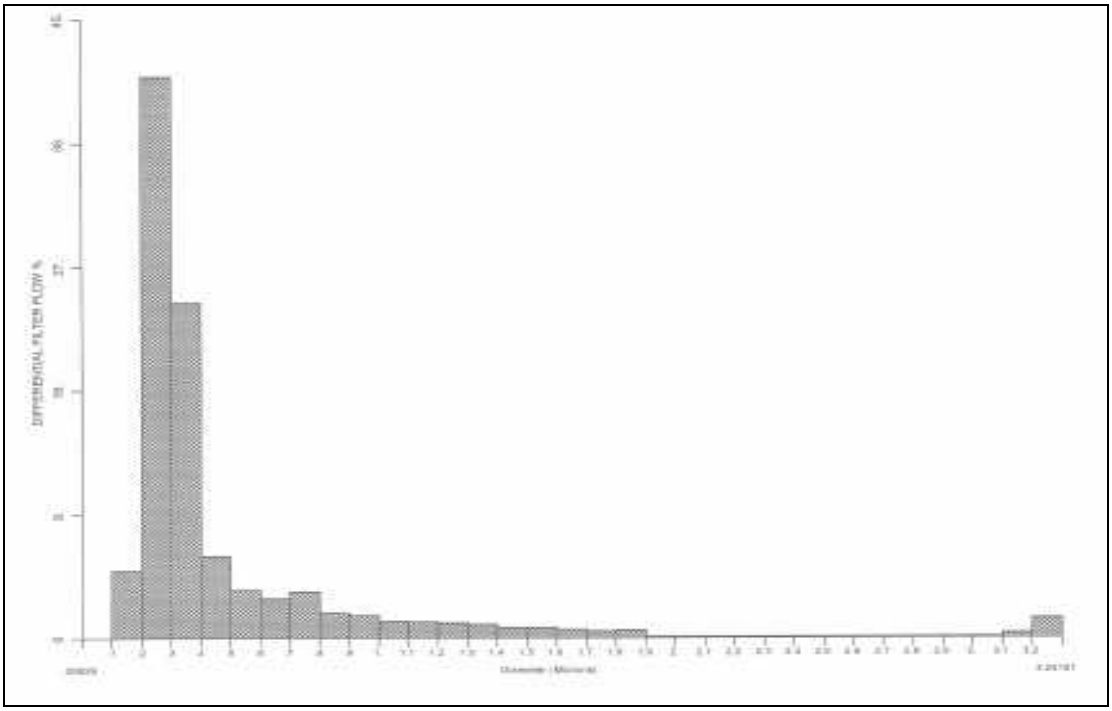

Fig. 3.1: Representative measurement of the pore size distribution of a microfiltration membrane.

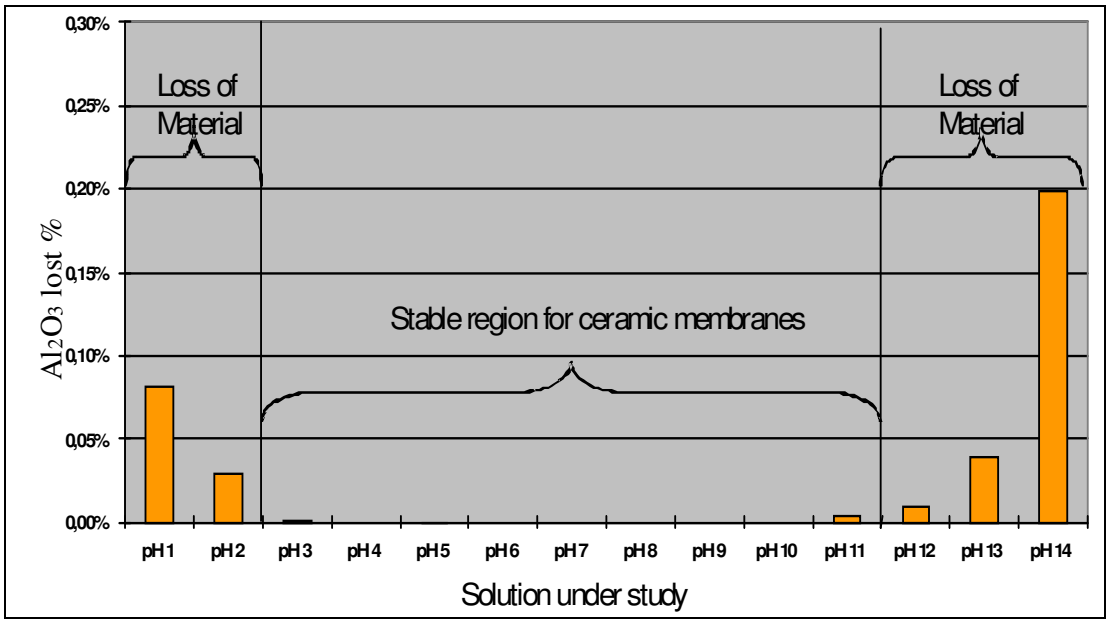

Fig. 3.2: Effect of pH of solution on integrity of submerged ceramic membrane.

3.1.2 Stability of membrane under process conditions

It is important that the employed membranes should not only be selective but also stable under the process conditions. One of the decisive factor could be their response to the prolonged exposure to the reaction medium under varying pH. Tests were carried out to check if there is any loss of the membrane material (Al_2O_3) when exposed to solutions having different pH. The reaction solution was water and the pH was adjusted using concentrated NaOH and HCl, which is a usual practice for fermentation processes. The results are graphically shown in figure 3.2.

3.2 The suitable adhesive

In addition to the membrane properties, the material selected for the module plays a decisive role for the integration of a submerged membrane system into a bioreactor. Particularly in this case, it is not only the ceramic membrane that has to be resistant to sterilisation (via autoclaving) but also the connections and sealing. While the connections for the module are made of stainless steel, a technique with a suitable adhesive for joining the parts between the membrane and the module has to be found (fig. 3.3).

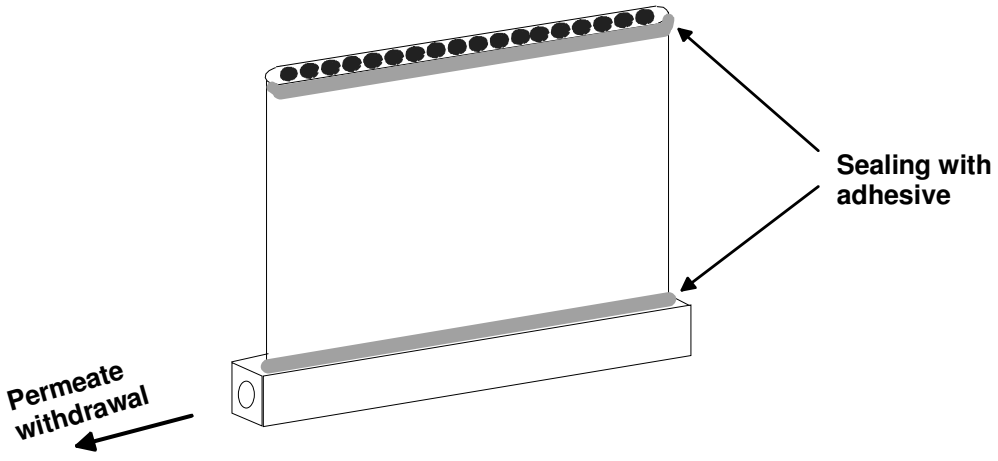

Fig. 3.3: Geometry of the flat-sheet membrane used in the tests and the employment of adhesive to integrate the membrane into the casing.

Table 3.1 Overview and evaluation of the adhesives under study.

Adhesive	Main component	Adhesion / ceramic & stainless steel	Resistance to		
			121 °C	HCl (1 mol/l)	NaOH (1 mol/l)
Supracraft PUR 566.0	Polyurethane	- -	- -	-	-
Suprasil 594 N	Silicone (Oxym-System)	- -	- -	-	-
Suprasil 590 E	Silicone (Acetate-System)	- -	- -	-	-
Bylapox 3125 A+B	Epoxy resin	- -	- -	-	-
Atmosit compact	1 component silicone	+	+++	+++	+++
Araldit 2021	Methacrylate	+++	- -	++	++
Araldit 2014	Epoxidharz	+++	-	++	++
Araldit XD 4510	Epoxidharz	++	-	+++	+++
Loctite 5910	Oxim-Silikon	- -	- -	-	-
UHU plus endfest 300	Epoxidharz	- -	- -	+	+
Hylosil	1-Komponenten-Silikon	-	+++	+++	+++
Scotch Weld 2216 B/A	mod. Polyurethan Härter: mod. Polyamin	++	- -	+	+
WEICON-Epoxyd-Minutenkleber	Epoxydharz Härter: Polyaminoamid	+++	-	++	++
WEICON Konstruktionskleber RK 1300	2-Komponenten-kleber	-	++	+	+
WEICON-Fast-Metal	Epoxydharz Härter: Polyaminoamid	+++	+++	+++	+++

Legends:

+++	very good
++	good
+	good to a certain extent
-	unsuitable
- -	unsatisfactory

A total of 15 different adhesives were tested and their suitability for autoclaving was examined. All the adhesives tested are shown in table 3.1. Since the module had to guarantee long-term operation with recurring sterilisation, the tightness of the connections was tested using pressure after each sterilisation process. From all the adhesives tested, only one could fulfil these requirements (chemical and thermal resistance) with complete satisfaction. The epoxy resin adhesive (WEICON Fast-Metal) from WEICON GmbH & Co. KG, Germany was the only one that could form a stabile and long-term connection between the ceramic material and the stainless steel. Furthermore, it proved to be resistant to pressure as well as the chemicals under study without losing the required properties even after several autoclaving processes. The same was used to prepare all the modules used in these studies.

3.3 Polymer coating on ceramic membrane

The method was suggested by ItN Nanovation AG, Germany and has been successfully used by them for sealing the ends of ceramic membrane. PFA 6900 RG dispersion (Dyneon GmbH, Germany) based on Polytetrafluoroethylene (PTFE) was used for the coating purposes. However, the method needs to be customized to the specific purposes and conditions. The polymer solution was filled in a pressurized vessel and the membrane to be coated was fitted in a stainless steel cross-flow module which is closed at one end (fig. 3.4).Hence, all the solution that was fed at one end must pass through the membrane matrix and leave the module at the permeate end. Thus, the membrane to be coated was subjected to a dead-end filtration mode. The operating pressure was adjusted up to 3 bars according to the pore size of the employed membrane and the concentration of the applied polymer solution. Solution was fed as long as there was permeate coming out of the membrane and hence the membrane was subjected to complete saturation with the polymer solution. The wet membrane was taken out and put in an oven for evaporating the water and fixing the polymer on its surface. First it was heated to the required temperature (150°C) in 180 minutes and then maintained at that temperature for 10 minutes. Then it was cooled down to room temperature in the next 30 minutes.

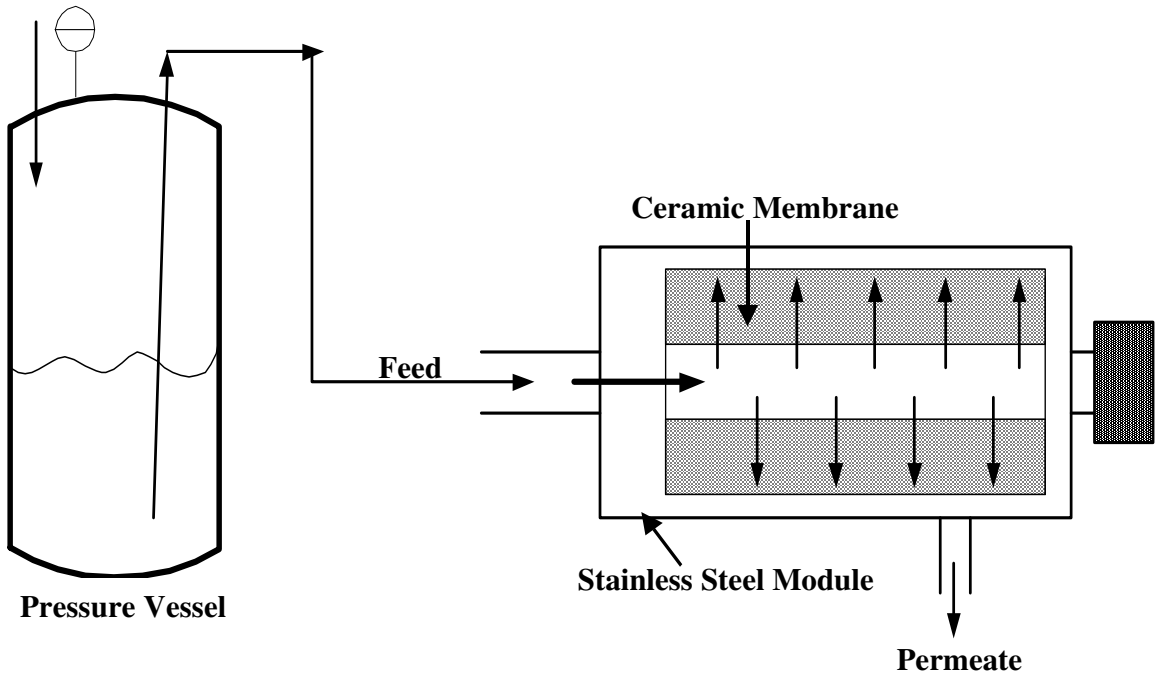

Fig. 3.4: Schematic diagram of polymer coating process, the ceramic membrane is enclosed in a cross-flow module and the feed flows through the membrane matrix outwards to the permeate exit.

The hydrophobicity of the coated membrane was tested by comparing the water flux of the coated and fresh membrane. To ensure that the decrease in water flux was due to enhanced hydrophobicity and not due to pore blockage during polymerization, the membranes were also tested with inert-gas flux which is not effected by membrane character (hydrophilic or hydrophobic) and depends only on the porous (free through) area available.

3.4 Analytical methods

3.4.1 Zeta potential determination

Zeta potential measurements were carried out using *Paar Physica* (Anton Paar, Austria). Measurements were carried out with NaCl, KCl and Na_2SO_4 - as electrolytes within a concentration range of 0.001-0.1 mol/l and a pH range from 2.5-11. The temperature of the electrolytic solution was maintained at 293 K. The cell needs to be rinsed before each measuring point and the pH value in the cell to be adjusted in approx. 90 min. The designated pH value was attained with HCl (NaCl and/or KCl solutions), H_2SO_4 (Na_2SO_4), NaOH (Na_2SO_4, NaCl) and KOH (KCl). Afterwards,

measurements were done in each direction, from which a middle value was computed.

3.4.2 Biomass determination

For analyzing the wet cell weight (wcw), unit sample was taken in an eppendorf tube and centrifuged at 7500g force and at 4°C for 2 minutes. The supernatant was thrown away and the tubes were weighed. The same tubes were then left open at 100°C in an oven for 24 hours and were weighed again for determination of dry-cell-weight (dcw). The standard calibration curve was obtained for OD value of the sample against the respective wcw and dcw. During experiments, only the optical density was measured at 600 nm and the corresponding value of biomass was calculated from the calibration equation.

3.4.3 Proteins analysis

For analysis of proteins bichinconinic acid (BCA) calorimetric method was employed. BCA Protein Assay kit from PIERCE, USA was used for the purpose.

3.4.4 Oil determination

About 20 ml of the reaction volume was taken out at regular intervals. After mixing well 5 ml was taken in a pre-weighted falcon tube. 5 ml of Hexane was added to it and after vigorous mixing by vortex; the falcon tube was put to centrifuge at 7500g force for 15 minutes at 18°C. After centrifugation, 1.5 ml volume was taken in pre-weighted eppendorf from the upper organic phase and left to evaporate for next 24 hours. After this the eppendorf tube was put for 6 hours at 60°C before being weighed to determine the oil.

3.4.5 Surfactants analysis

About 20 ml of the reaction volume was taken out at regular intervals. After mixing well 5 ml was taken in a pre-weighted falcon tube. 5 ml of Hexane is added to it and after vigorous mixing by vortex, the falcon tube was put to centrifuge at 7500g force for 15 minutes at 18°C. From the lower aqueous phase 3 ml volume was taken in a separate falcon tube to which 30 µl of H_3PO_4 was added to protinify the rhamnolipids. 4 ml ethylacetate was added and after vortex, the falcon tube was put to centrifuge

again at the same force but at 4°C. After centrifuge, 1.5 ml was taken from the upper organic phase and left to evaporate. The rhamnolipids were analyzed from the pallets by HPLC (Agilent 1100, Agilent, Germany; 70% ACN, 265 nm, 25°C).
For quick analysis and qualitative comparison, the concentration of surfactants was analyzed using 'Dr. Lange Schnelltest für Tensides' (HACH LANGE GmbH, Germany).

3.4.6 Alginate analysis

The analysis was done by high performance liquid chromatography (HPLC). The eluent was mili-Q water at 30°C and the flow rate was set at 1.0 ml/min. The column used was SEC 2000 (Waters, USA) With Fluorescens detection (Shimadzu, Japan).

3.4.7 Viscosity Measurement

Measurements were carried out using capillary viscometer (SCHOTT-instruments GmbH, Germany) at room temperature.

3.4.8 Droplet size measurement for emulsions

For this analysis, the oil droplets in a stable emulsion were assumed as insoluble particles in a solution. Thus they were treated like a non-interacting entity. 'LS100 particle size counter' (COULTER, USA) available at Department of Process Technology, University of Saarland was used for the purpose. The determination is based on the flow of sample volume through standard channels and the result is reported in terms of percentage of total volume passed through that particular channel of specific pore size. Thus, the sample size is not a factor and the fraction of total particles falling under a particular size is calculated from the differential volume results for the respective channels.

3.4.9 Contact angle measurement

A small droplet of test solution is put on the surface of the membrane under study and the spreading of the liquid is monitored with a micro camera attached to a computer for online data recording. Depending on the organic/aqueous nature of the fluid and hydrophobic/hydrophilic nature of the surface the spreading could be fast or slow. In any case there is a period when the contact angle between the surface and

the droplet is constant, and this value is taken for analyzing the interaction. For example, increase in contact angle of a droplet from an aqueous solution would mean that subsequent surfaces are more and more hydrophobic and vice versa. 'DSA100' drop shape analyzer (Krüss GmbH, Germany) was used for the purpose. Though it is more of a qualitative method rather than quantitative, it is important to analyze the interaction between a liquid and a surface — in our case the solution to be filtered and the membrane.

3.4.10 Polyhydroxyalkanotaes (PHA) anaylsis

The Sample from the bioreactor was centrifuged at 7500g force to separate the biomass. Qualitative analysis was performed with Thin Layer Chromatography (TLC) on 0.25 mm silica gel plates using a mixture of chloroform:methanol:acetic acid 65:25:4 v/v/v as developing solution. Plates were visualized with α-naphthol sulfuric acid soltion and heating at 100°C. Quantitative analyses were carried out by Gas Chromatography (GC) on the methyl esters of the extract. Samples were methylated with diazomethane, diluted with ether and injected on a Shimadzu GC-14A (Shimadzu, Japan).

3.4.11 Lysine anaylsis

The quantification of the amino acids in the cell medium was done with HPLC (Agilent 1100, Agilent, Germany). The cell medium was diluted 1:2 with a solution containing 225 µM of α-Aminobutyric Acid (ABU). ABU acts as an internal standard for the quantification. The separation was via a guard cartridge and a Gemini column (Phenomenex, Germany) and was carried out at a flow rate of 1 mL/min at a column temperature of 40°C using 40 mM NaH2PO4 (eluent A, pH 7.8, adjusted with NaOH) as polar phase and a Acetonitrile/Methanol/Water mixture (45/45/10) as non-polar phase (eluent B). The amino acids are pre-derivatized with o-phtaldialdehyde (OPA) and mercaptoethanol (Roth 1971) subsequently followed by 9-fluorenylmethyl chloroformate (FMOC-Cl) with 3-Mercaptopropionate (0.5% in 0.5M Bicin) for Proline. The detection of the amino acids is via a Florescence detector (Agilent, Germany) with 340 nm Excitation and 450 nm Emission for the primary amines and changed at 43.5 minutes to 266 nm Excitation and 305 nm Emission for the detection of proline.

The peaks are manually integrated, and the quantification is done knowing the dilution and the concentration of the internal standard.

3.5 Materials used for study

All chemical were bought from Sigma-Aldrich, Germany unless otherwise mentioned. Ceramic membranes were bought from different suppliers, which are mentioned at their respective appearance in the manuscript. All stainless steel fittings for the membrane modules were bought from Best GmbH, Germany.

4 Biomass retention and product recovery: Yeast fermentation

Yeast is an attractive host for the production of heterologous proteins. Unlike prokaryotic systems, their eukaryotic subcellular organization enables them to carry out many of the post-translational folding, processing and modification events required to produce "authentic" and bioactive mammalian proteins. In addition, they retain the advantages of a unicellular microorganism, with respect to rapid growth and ease of genetic manipulation.

4.1 Methylotropic Yeast: *Pichia pastoris*

During the last 20 years, the methylotropic yeast *Pichia pastoris* has developed into a highly successful system for the production of a variety of heterologous proteins. The increasing popularity of this particular expression system can be attributed to several factors, most importantly:

1) the ability of *P. pastoris* to produce foreign proteins at high levels, both intra-cellularly as well as extracellularly;
2) the simplicity of techniques needed for the molecular genetic manipulation of *P. pastoris* and the similarity of techniques with those for *S. cerevisiae*;
3) the capability of performing many eukaryotic like post-translational modifications;
4) and the availability of the expression system as a commercially available kit.

Main feature of *Pichia pastoris* is its similarity to *Saccharomyces cerevisiae* (also known as baker's yeast). *S. cerevisiae* is well studied as a model organism for biology and has been used by mankind for various purposes throughout history. The two yeast species (*Pichia, Saccharomyces*) have similar growth conditions and tolerances, and thus the culturing of *P. pastoris* can be readily adopted by labs without specialist equipment.

Pichia has a high growth rate and is able to grow on a simple, inexpensive medium. Cell densities up to 450 g/l wet cells have been reported, thus making it a favorable choice for high cell density fermentations. *Pichia pastoris* has two alcohol oxidase genes, AOX1 and AOX2, which have a strongly inducible promoter. These genes

allow *Pichia* to use methanol as a carbon and energy source, and hence provide unique advantages to be chosen as a host [Daly and Milton, 2004].

4.2 Objective of the study

The objective of this study was to enhance the biomass density inside the bioreactor and hence improve the productivity of the bioprocess. This was planned by integrating a submerged membrane system into the bench scale bioreactor for continuous removal of the product, secreted into the medium by the recombinant *pichia pastoris*, while retaining the high amount of biomass. In this method the reaction volume is maintained by addition of fresh substrate, the biomass grows further while the product inhibition (if any) is checked by continuous removal of the product. The strain is developed by our project partners at the Institute of Technical Microbiology in Technical University Hamburg-Harburg, Germany. The aim is to produce an extremophilic Lipase which has vital industrial applications in food, detergent, and pharmaceutical sectors [Houde et al., 2004]. The details of the biological work are not discussed here. The fermentations were carried out following the 'pichia fermentation process guidelines' published by *invitrogen*.

The plan of action comprised the selection of suitable membrane, its integration into the bioreactor and then optimization of the operation which is discussed in the following sections.

Fig. 4.1: Image under light microscope and measurement of a *Pichia* cell.

4.3 Selection of suitable membrane

The membrane employed for this purpose should be resistant to the cells and at the same time permeable to the desired product. Measurements for particle size distribution as well as microscopic tests showed that the yeast cells, which were to be separated, were spherical and had an average size of roughly 3 to 6 µm (fig. 4.1). The desired protein had a molecular size of less than 100 nm.

4.3.1 Optimal pore size for membrane selection

As the difference between the size of cells to be retained and the product to be permeated was quite significant, it was decided to test the whole range of membranes available with pore size bigger than that of the given protein. A total of five different membranes generously supplied by ItN Nanovation AG, Germany, with an expected average pore size (according to manufacturer's details) between 200 and 800 nm were screened using the flow-pore method and pure water permeability. The results are summarized in table 4.1.

Table 4.1: Characterisation results of the membranes under study.

N0.	Coating (manufacturers' data)	Bubble Point [µm]	Average pore ∅ [µm]	Water permeability [l/(m²·h·bar)]
1	200 nm	0.80	<< 0.07	65
2	200 nm	0.89	<< 0.07	110
3	300 nm	0.44	0.10	500
4	300 nm	0.81	0.19	600
5	800 nm	5.08	0.37	790

As can be seen in table 4.1, membrane no. 4 provided the best separation properties for the current study. With an average pore size of 0.19 µm and a water permeability rate of 600 l/(m²·h·bar), this membrane proved to be more favorable than membrane no. 5 because it displayed a considerably narrower pore size distribution. The bubble point measured for membrane no. 5 was roughly 5 µm, which could not ensure total

retention of the yeast cells. It is interesting to note that membranes provided under similar category with respect to average pore size showed such a big difference from their reported characteristics. This could be attributed to the fact that these samples were provided from the membranes under development phase at the company, and later on the supply was satisfactory as will be seen in the coming chapters. Nevertheless, it is possible to test (characterize) the membranes for their suitability to a particular application.

4.3.2 Test for membrane fouling: zeta potential measurement

The adsorptive interaction of medium components with the membrane frequently cause membrane fouling, resulting in a considerable reduction in permeate flux with filtration time [Hunter, 2001]. Hydrogen bonds, Van-der-Waals' forces and hydrophobic interactions are conducive to adsorption while like charges have an inhibiting effect [Sartor, 2006]. To record possible electrostatic interactions between the yeast under consideration and the selected membrane (no. 4), the zeta potential of the yeast (*Pichia pastoris*) as well as of the ceramic membrane was determined over a wide pH range.

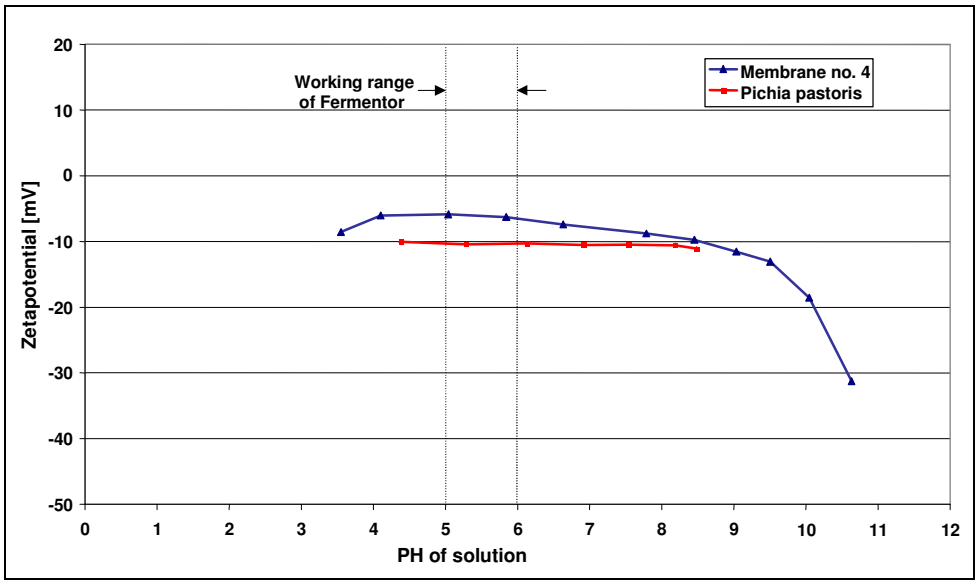

Fig. 4.2: Zeta potentials of the yeast and membrane no.4 for a wide pH range.

Figure 4.2 shows the zeta potential measured for the ceramic membrane and the yeast. As can be seen, the potentials measured for both surfaces were in the same range of roughly -10 mV between the pH values of 5-6, which was the relevant pH

range for the current fermentation. Judging from this, it could be concluded that the membrane is less prone to fouling during yeast filtration because of the like surface charge and the related mutual repulsion.

4.3.3 Retention efficiency of the selected membrane

Based on preliminary membrane screening, lab-scale filtration tests were then performed under approximately real conditions to record the retention efficiency of the selected membrane for the yeast cells as well as the permeability of the desired protein through the membrane.

For this purpose, a filtration unit consisting of a reactor vessel and the necessary filtration equipment (pumps, pressure recorder etc) was set up. The membrane was submerged in the reactor and the permeate was withdrawn using a peristaltic pump. The membrane permeate flux was then measured gravimetrically using a balance and determined as a function of filtration time. Membrane permeability [l/(m²·h·bar)] could be determined by simultaneous online recording of the transmembrane pressure (TMP) and weight of the collected permeate.

- **Yeast retention**

In the first test series, the retention of the yeast cells was determined by using the selected membrane (no. 4, table 4.1). For this purpose, yeast was cultivated in several shaking flasks in order to obtain a sufficient volume for the filtration tests. The biomass concentration (WCW) in the test bioreactor was constantly increased in five successive test series.

The first tests were conducted using a wild strain of *Pichia pastoris* in biomass concentrations of up to 40 g/l, while concentrations from 40 g/l to 255 g/l were simulated using commercial baker's yeast. Membrane integrity was tested by determining the optical density of the permeate and was verified via the rheological determination of viscoelasticity [Chmiel, 1991]. The results were corroborated with microscopic examination.

Table 4.2: Retention of the yeast cells at different biomass concentrations.

Test Nr.	Concentration WCW [g/l]	OD_{600} Reactor	OD_{600} Permeate	Retention [%]
1	23	7	0.003	99.96
2	44	21	0.006	99.97
3	82	32	0.010	99.97
4	177	90	0.012	99.99
5	255	141	0.021	99.99

In the test range under investigation (up to 255 g/l wcw), no difference in retention capacity was detected for the membranes. Regardless of the biomass concentration in the reactor, retention was always in the range of 99.9% and could thus be described as total retention (table 4.2, fig. 4.3).

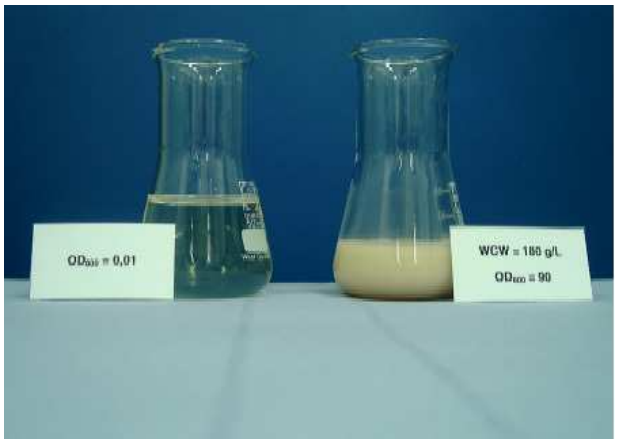

Fig. 4.3: Optical examination of permeate quality (test no. 4, table 4.2), on the right is sample from the culture mixture and on the left from the permeate.

- **Protein retention**

To record the permeability of the membrane for the given protein, filtration tests were performed using a model protein solution (lipolase from Novozymes, Germany) at different pH values (fig. 4.4). This was done in order to check if any adsorption of the enzyme to be isolated occurs on the ceramic membrane under study. To this end, a specified quantity of lipolase was put into the reactor and filtered by the ceramic membrane over a longer duration of time. Samples were taken from the reactor

(feed) and the permeate at regular intervals and their protein content was compared. The tests were conducted for equal concentrations at pH 4 and pH 7.

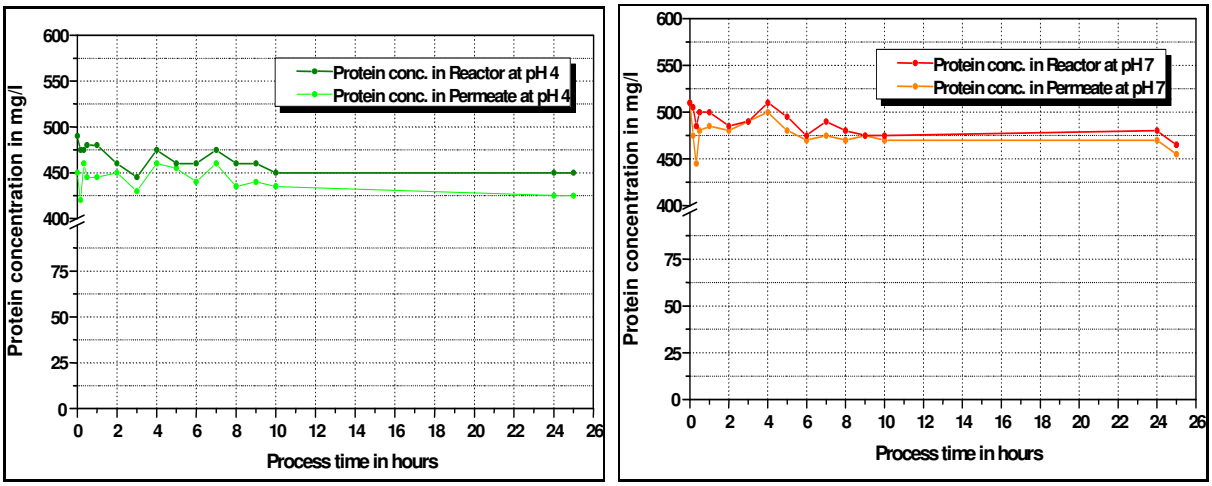

Fig. 4.4: Protein concentration in the bioreactor and in the permeate during filtration of a lipase solution (pH 4, pH 7) using membrane no. 4.

As illustrated in figure 4.4, no significant retention of lipolase by the membrane could be detected during both the tests. During the filtration period, the loss of protein in the permeate was constantly in the range of < 2%. Thus, adsorption and other interactions between the protein and the membrane under study could be ruled out.

4.4 Optimization of filtration mode

During membrane filtration the decline in flux is commonly connected to two phenomenon: concentration polarization and fouling. The level of membrane fouling is dependent on the feed suspension properties (particle size, particle concentration, pH, ionic strength), membrane properties (hydrophobicity, charge, pore size) and hydrodynamics (transmembrane pressure, cross-flow velocity) [Kylloenen et al., 2005]. In order to control fouling and to ensure a continuous and high permeate flux through the membrane in this case, the operating mode of the membrane (with periodic back flushing or with periodic breaks in operation) needed to be optimized. Firstly, membrane operation with periodic breaks in operation was investigated. One decisive advantage of this operating mode compared to the mode with periodic back flushing is that a part of the permeate does not have to be re-filtered (in case of back flushing the amount used in back flush has to be filtered again), resulting in a clear increase in the net permeate flux of the membrane.

As shown in figure 4.5, the breaks in operation have a considerably positive effect on the permeate flux. After using the membrane for filtration purposes only – and hence operation without any fouling control measures – which resulted in a drop in permeate flux (at constant transmembrane pressure) of more than 50%, the membrane process was carried further with periodic breaks in operation. After the break, filtration flux increased by four times for a short period before a stable value of roughly 100 l/(m²·h) was re-established.

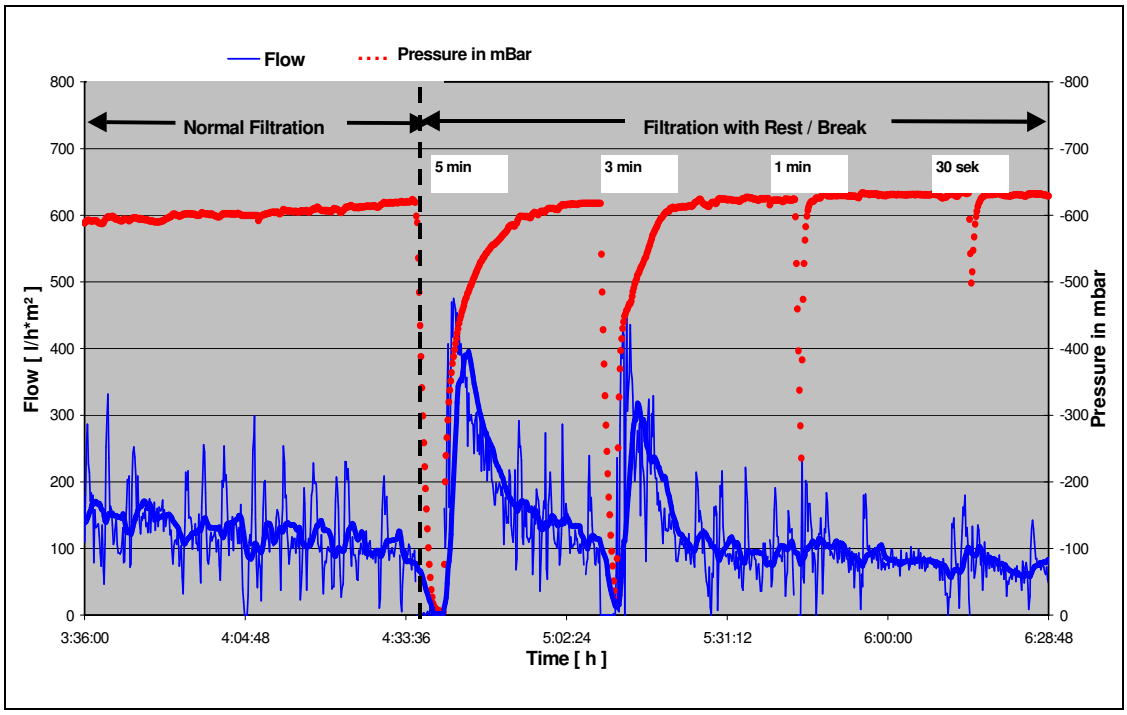

Fig. 4.5: Filtration test with periodic breaks to determine the best possible operating mode of the membrane for high biomass density (245 g/l, wcw) filtration. The breaks were applied after 30 minutes of filtration and the duration of breaks was decreased step-wise from 5 minutes to 30 seconds.

Figure 4.5 also shows that the effect of breaks on filtration operation varies with the duration of breaks. During the long-term test, breaks of different durations were tested. The lengths of the break (after 30 minutes filtration time respectively) ranged from 5 to 30 minutes. From this test it was determined that the filtration process had to be stopped for at least 3 minutes (after 30 minutes filtration) in order to remove as much layer of yeast cells from the membrane as possible, and thus generate any significant improvement in permeate flux. This positive effect of successful fouling control has been described several times in literature on cross-flow operations [Marrot et al., 2005] and is the result of an increase in the shear forces generated by

breaks in permeability (i.e. by stopping the filtration process). This increase is due to interruption of the convective mass transfer on the membrane surface. In the present case, the shear forces generated by the stirrer were used to remove the fouling layer that had formed on the membrane surface.

In further test series, the effect of turbulence (induced by the stirrer in the bioreactor) and therefore the subsequent membrane flux generated was investigated in relation to the optimized breaks in operation. It was observed that the minimum amount of stirring (500 rpm) needed for the process and thus the turbulent flow conditions already existing in the bioreactor could guarantee an optimum membrane flux. Tests involving a much lower degree of stirring in the bioreactor (the number of revolutions for the stirrer was set to 15 times less than that required for the fermentation process) did not show any negative effect on hydraulic efficiency of the membrane.

An assessment of the membrane that had undergone operation with the optimum settings in a long-term test showed that, at least optically, no significant fouling layer could be detected on the membrane (fig. 4.6). Thus the prediction from zeta-potential test was confirmed. Moreover, the membrane did not display any discolouring due to biofouling, the reason for which could be the absence of extracellular polymeric substances (EPS). Such deposits of micro-organisms on wet surfaces do not occur with yeast.

Fig. 4.6: Optical examination of a membrane after a long-term test (filtration time 30 minutes; periodic break in operation 3 minutes).

Fig. 4.7: The single-channel membrane.

4.5 Alternative concept to flat-sheet membranes

The tests showed that these module designs, involving the use of ceramic flat-sheet membranes, are only suitable for fermenters of approximately more than 5 litres operating volume. It is also not possible to integrate a suitable module in very small fermenters (< 3 l) in a technically effective manner because of the dimensions of flat-sheet membranes and their respective fittings.

For this reason, it was planned to replace the multichannel flat-sheet membrane by a single-channel cylindrical membrane (fig. 4.7). This membrane was also produced by ItN Nanovation AG based on the intermediate results. The single-channel membrane is made of the same basic material (Al_2O_3) as the previously used multi-channel flat-sheet membrane and has the same separation properties. The only difference is that unlike the flat-sheet membrane they have their decisive thin layer on the inner side. These cylindrical membranes are normally used for cross-flow filtration where the permeate flows through the support matrix in the outward direction. However, as the supporting matrix on the outer side has an average pore size of 600-800 nm, hence the membrane is suitable for the size of yeast under investigation.

Similar to the multi-channel flat-sheet membrane, the epoxy resin adhesive (WEICON Fast-Metal) ensured a stable connection between the ceramic membrane and the stainless steel fittings. Moreover, the single-channel membrane could be fixed through the top or the bottom of the fermenter as both parts were fitted with connections. For stability reasons, the membrane was fixed onto the bottom of the fermenter (fig. 4.8, left). Furthermore, due to its smooth geometry the cylindrical module offers less resistance to the flowing medium as compared to the flat-sheet modules. Hence, fouling at the surface could be better avoided with this design.

In tests with the laboratory fermenter (2 l operating volume), the use of the single-channel membrane and the selective arrangement in the fermenter under real operating conditions proved to be successful. Data regarding lipase production and productivity of the bioprocess could not be revealed here. The biomass (approximately 300 g/l, wcw) could be retained completely inside the bioreactor, and a clear permeate was collected through the membrane (fig. 4.8, right) using a simple peristaltic pump. There was no loss of lipase due to adsorption on the membrane,

and no problem due to membrane fouling was observed. Thus, a semi-continuous and sterile operation was successfully achieved.

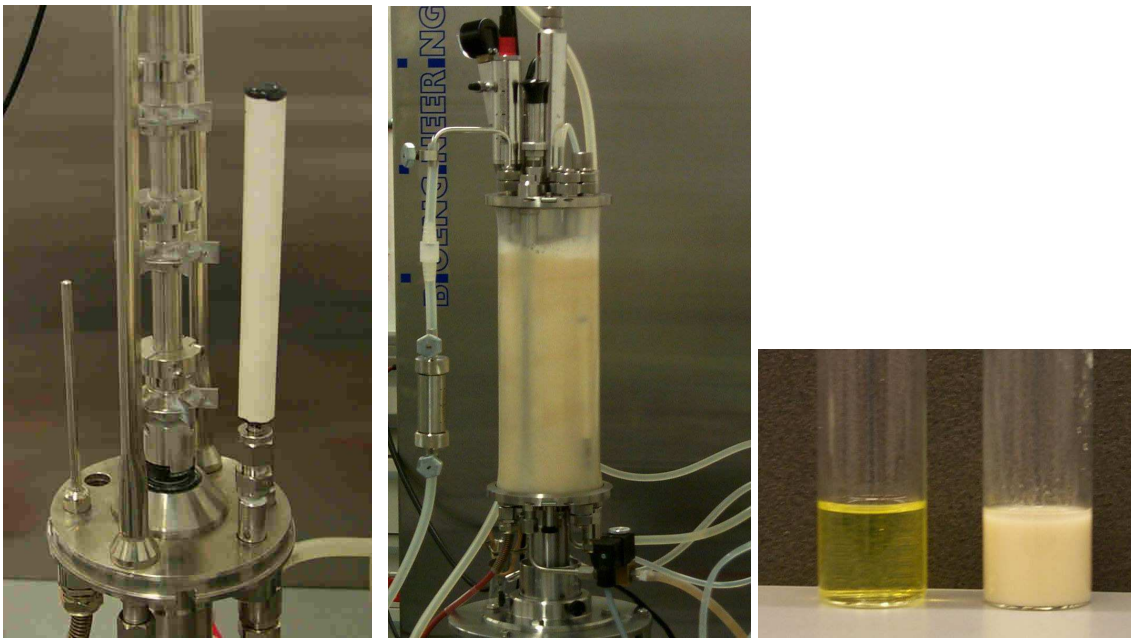

Fig. 4.8: The single-channel membrane installed in a laboratory fermenter with an operating volume of roughly 2 l (left and middle); Comparison of the filtered solution free of solids and the biomass (right).

4.6 Metabolites production with *Schizosaccharomyces pombe*

4.6.1 Fission Yeast : *Schizosaccharomyces pombe*

Schizosaccharomyces pombe, also called "fission yeast", is a species of yeast that is used as a model organism in molecular and cell biology. It is a unicellular eukaryote, whose cells are rod-shaped. Cells typically measure 3 to 4 μm in diameter and 7 to 14 μm in length. *S. pombe* is almost as easily cultured and manipulated as baker's yeast. It has been well characterized in the field of classical and molecular genetics, its nuclear genome has been sequenced, and it is an alternative fungal model system, comparable to that of the yeast *Saccharomyces cerevisiae*. It became the sixth model eukaryotic organism whose genome has been fully sequenced. This has fully unlocked the power of this organism, with many genes homologous to human disease genes, including diabetes and cystic fibrosis, being identified. It also offers numerous advantages to studies of DNA replication and provides an excellent model system between budding yeast and metazoans. It is necessary, however, to improve the system further for the production of low-cost chemicals and commodities, so that

the host becomes more economical and productive and can be widely used for the production of different molecules [Giga-hama et al., 2007].

4.6.2 Objective of the study

At PomBiotech GmbH, Saarbruecken, Germany an overexpressing strain of *S. pombe* (CAD 65) has been developed for expression and secretion of two human proteins: P450- CPR and P450-Cyp2B6, present in cynobiotic metabolizing system in human lever. The metabolites produced by the modified strain are of interest for pharmaceutical as well as analytical purposes [www.pombiotech.de]. The conventional process is a two-step batch process where the biomass is grown in the first step, the cells are centrifuged and washed repetitively and then introduced into another medium where biotransformation of specific substrates is carried out to produce specific metabolites. The details of the growth and biotransformation medium as well as the substrates and the produced metabolites could not be revealed here. However, it is to be noted that biotransformation is directly related to biomass density. Thus, the aim of this study was to increase the biomass concentration in the first step so that the subsequent biotransformation could be improved. An average biomass growth of 9 g/l was observed after 48 hours conventional batch process employing a 3-liter bioreactor with 1.5 liters working volume.

Based on our earlier success with *P. pastoris* fermentation (section 4.5), it was decided to employ ceramic membranes inside the bioreactor. The strategy was to draw out half of the reaction volume after the consumption of the initially fed substrate and then to make up the reaction volume with fresh substrate and salts solution. Thus in principle, a repeated batch process has been proposed. The biomass was concentrated by employing submerged membrane filtration, and the subsequent batch was started with the high biomass density achieved in the previous step. Thus, the whole process could be carried out within one set up and without risking the sterility, which is crucial as the cells would later be used for production of human metabolites.

4.6.3 Membrane process 1

As the bioreactor was small (3 liters) and the total working volume was only 1.5 liters, monochannel membrane was the preferred choice, as has been discussed earlier (section 4.5). Cylindrical-ceramic membrane support (ID=8mm, OD=14mm) with average pore size between 0.5-0.6 µm was used (ItN Nanovation AG, Germany). As the size of the *pombe* cells is between 3-4 µm, the ceramic support was suitable enough for the filtration and chosen over a complete membrane (with thin layer) of similar pore size due to cost factor. The membrane module was fitted into the bioreactor from the top and the total filtration area available was approximately 31 cm^2. The amount of substrate in the solution was regularly monitored by analyzing the samples collected for biomass determination. When the concentration of the substrate was significantly low, suction was performed using a peristaltic pump (TMP < 400 mbar) and 50% of the culture volume was drawn out. Then, fresh medium was fed to make up the reaction volume and the next suction was performed when a significant fall in the substrate concentration was observed. The permeate was analyzed for the presence of cells and the unused substrate.

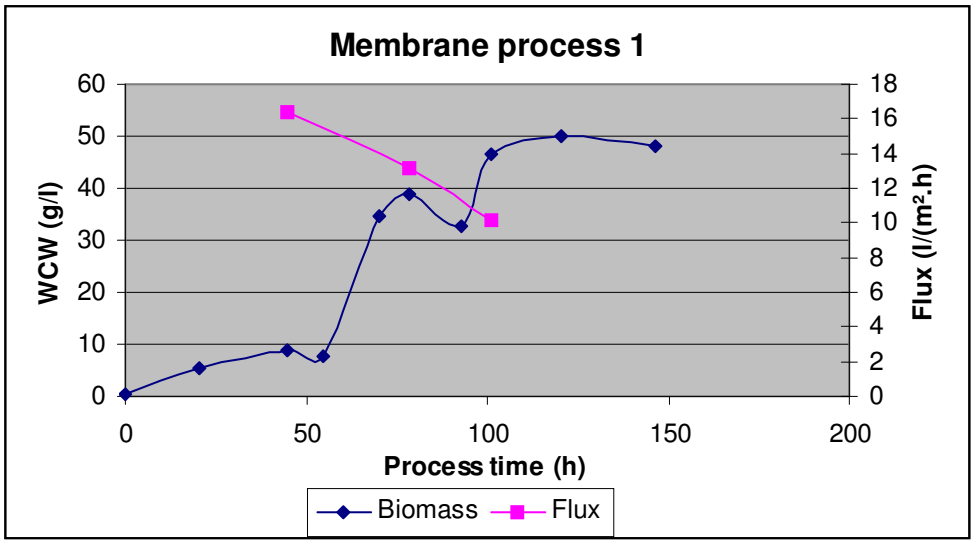

Fig. 4.9: Biomass and flux from membrane process 1: the filtrations were carried out 3 times and the flux decreased in subsequent filtration due to higher cell density inside the bioreactor.

As can be observed in figure 4.9, a total of about 50 g/l (wcw) biomass was produced in about 150 hours of fermentation. During the process the filtration was carried out three times and accompanied by an observable reduction in flux. This decrease could

be attributed to the fact that subsequent filtration was performed at a higher cell density inside the medium. High biomass resulted in a denser cell layer formation on the membrane surface and hence increased the overall resistance to flow. The final concentration of cells before introducing them to the biotransformation medium had to be performed by centrifugation, as the medium used for growth needed to be washed out completely before starting the biotransformation. Nevertheless, in the same culture volume almost five times higher cell density could be achieved, and hence the number of biotransformation units were significantly more as compared to the conventional batch process.

4.7 Development of new membrane modules

Although the biomass obtained with membrane process 1 was significantly higher than the usual batch process, the long duration of membrane integrated-fermentation was not satisfactory for practical reasons. To replace the conventional batch process with membrane-integrated process, it was required to enhance the flux. To achieve this, filtration area was increased; and given the scenario, this method is to be considered the only feasible solution as increasing the suction pressure (TMP) would only increase the formation of cell layer over the membrane surface. Moreover, due to limited space available inside the bioreactor it was not possible just to integrate more membranes, but a new design needed to be developed. The new module should offer more filtration area and at the same time the available space and long term stability of the employed membranes should be taken into account.

4.7.1 The Λ module

Two cylindrical pieces (5 and 7 cm long) of the same ceramic support used before were joined together via a bend steel tube using WEICON Fast-Metal adhesive. Two such sets were integrated using Swagelok® stainless steel fittings (Best GmbH, Germany). Total filtration area available with this module was approximately 105 cm^2 and the whole module could be comfortably fitted inside the stainless steel cage of the bioreactor (fig. 4.10). Moreover, the **Λ** sets were placed between the impellers of the reactor thus ensuring maximum turbulence over the membrane surface during

the process. This was important to avoid the cell layer formation over the membrane surface which otherwise would have resulted in significant loss of flux.

Fig. 4.10: The **Λ** module: the available filtration area was approx. 105 cm^2. The membranes could be fitted safely inside the stainless steel cage of the bioreactor. Cylindrical membrane attached via a steel tube (above) and the whole module fitted into the bioreactor (below).

The results of the membrane process carried out with the **Λ** module are shown in figure 4.11. It is to be noted that for the process, a 5-liter bioreactor was made available and the total working volume was kept at 3 liters. A total of about 50 g/l (wcw) biomass was obtained which the same as was before. The reduction in flux with subsequent filtration showed a similar pattern as observed earlier. However, this

process was carried out for 96 hours while the previous process was carried out for 150 hours. Moreover, the reaction volume was 3 liters, 1.5 liters more than what was used in the membrane process 1. Thus higher total amount of biomass and hence higher biotransformation was achieved in less total process time with the new submerged membrane system.

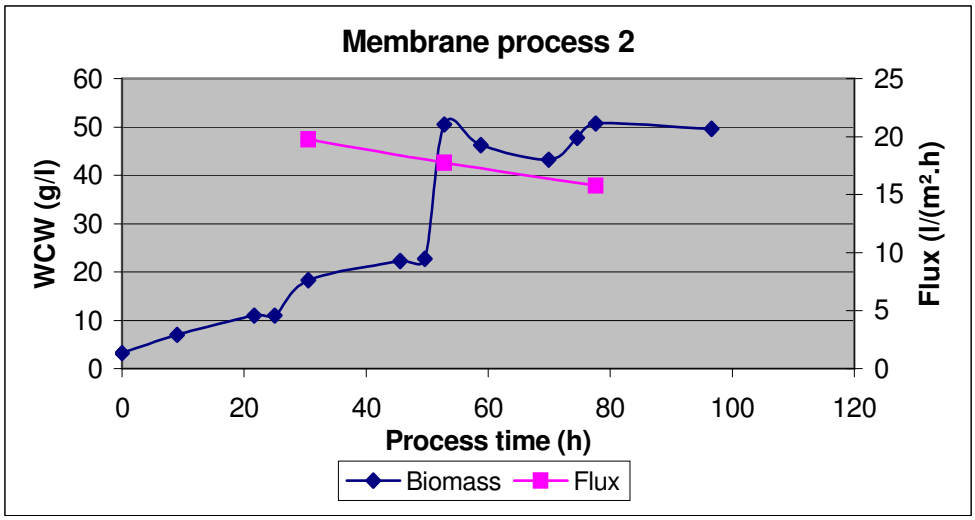

Fig. 4.11: Biomass and flux from membrane process 2 with **Λ** module: the filtrations were carried out 3 times and the flux decreased in subsequent filtration due to higher cell density inside the bioreactor.

4.7.2 The Π module

The total filtration area as well as the total flux achieved with the **Λ** module was significantly higher as compared to a single membrane system. However, there were certain limitations with respect to long-term use of the **Λ** module. As the two membrane pieces in the **Λ** set were fixed permanently, any damage faced with either of the membranes would have resulted in the whole set to be changed. To circumvent this, it was aimed to design a new module where all the employed membranes are independent but still integrated. Different standard Swagelok® stainless steel fittings (Best GmbH, Germany) were fitted with each other to obtain a new module where 4 membrane pieces could be integrated independently (fig. 4.12). The membranes could be placed parallel or at a certain angle to each other, thus could be adjusted according to the available space and working volume inside the bioreactor. Moreover, as all the parts used to prepare this module were standard fittings, the extension or replacement was not an issue. Four cylindrical ceramic

support pieces, each eight cm long, were used and the total filtration area made available with this module was 140 cm^2.

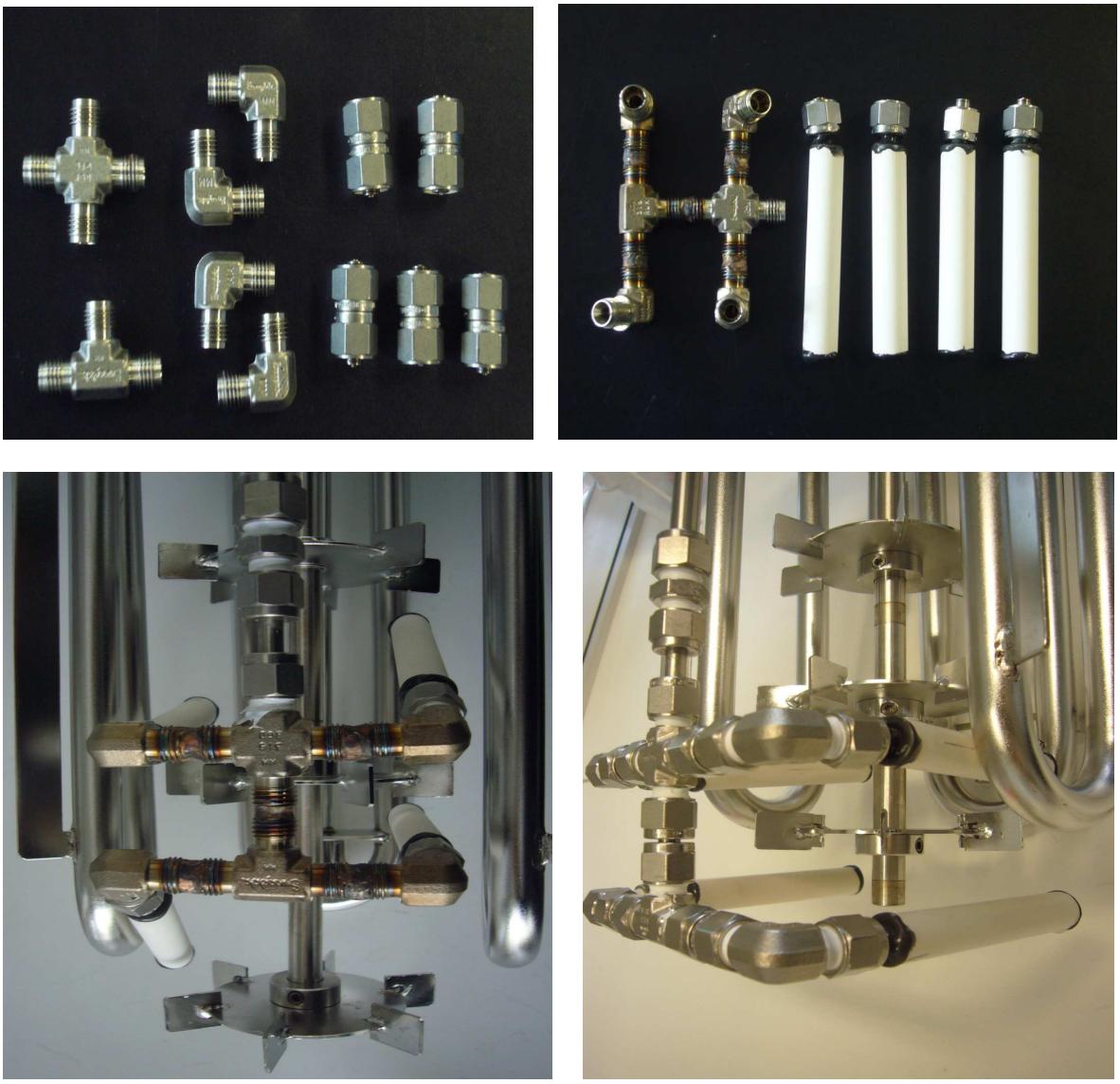

Fig. 4.12: The **Π** module: the available filtration area was approx. 140 cm^2. The membranes could be fitted safely inside the stainless steel cage of the bioreactor and the integrated membranes could be rotated to adjust to the available space. Stainless steel fitting and the resulting module and membranes (above) and the whole module fitted into the bioreactor (below).

The results of the membrane process carried out with **Π** module are shown in figure 4.13. A total of about 82 g/l (wcw) biomass was obtained in 99 hours with suction performed twice. It is to be noted that with this module approximately 65% of the reaction volume was drawn out as compared to 50% that was drawn out in earlier membrane processes. The total process time was similar to that in membrane process 2 with **Λ** module, but nearly 60% increase in biomass concentration was

achieved. Moreover, the new module offered flexibility of customization to the specific case with respect to the available space in the bioreactor and the volume to be filtered. The same module could be shortened or extended by simply removing or adding the standard fittings.

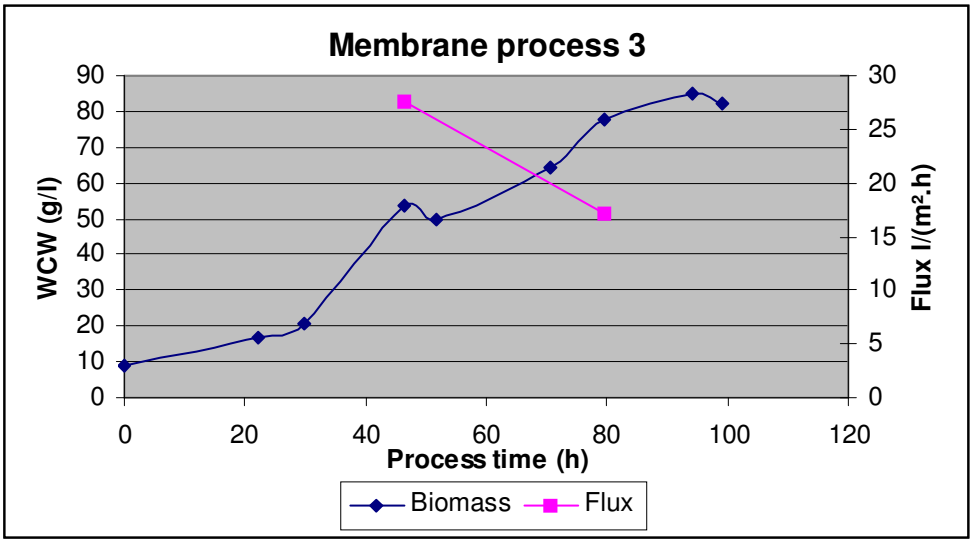

Fig. 4.13: Biomass and flux from membrane process 3 with **Π** module: the filtrations were carried out 2 times and the flux decreased in subsequent filtration due to higher cell density inside the bioreactor.

4.8 Conclusion and outlook

Employment of submerged ceramic membrane systems for high cell density yeast fermentation has been successfully shown. Investigations have been carried out with the most commonly used yeast: *Saccharomyces cerevisiae*, *Pichia pastoris* and *Schizosaccharomyces pombe*. The interaction of membranes with the yeast cells did not show any negative effect, which is very important for acceptance of the developed method for bioprocesses being developed for sophisticated purposes such as pharmaceuticals. Furthermore, development of different modules has been discussed according to specific needs of the bioprocess as well as depending on the available space within the bioreactor. Investigations are planned for production and recovery of an antibody from high cell density *S. pombe* fermentation employing the **Λ** and **Π** modules. The methodology is suitable to scale-up and should be investigated at pilot scale.

5 Biomass retention and product recovery: Bacterial fermentation

Bacteria are unicellular microorganisms. They are typically a few micrometers long and can have many shapes such as spheres, rods, and spirals. Owing to their ability to grow quickly and the relative ease with which they can be manipulated, bacteria are the workhorses in the fields of molecular biology, genetics and biochemistry. The ability of bacteria to degrade a variety of organic compounds is remarkable and has been used in waste processing and bioremediation. In the chemical industry, bacteria are most important for the production of enantiomerically pure chemicals for pharmaceutical or agrochemical applications. Researchers can alter most of the genetical make-up of the bacteria to serve as "factories" for synthesizing DNA and/or proteins, which can then be produced in large quantities using the industrial fermentation processes [Shiloach and Fass, 2005].

5.1 Background and objective of the study

This project is designed to develop a cost-effective production process for biosurfactants based on renewability of the raw material. Surfactants are wetting agents that lower the surface tension of a liquid, thereby allowing easier spreading of the dispersed phase and hence have vital applications in the oil and detergent industry [Ochsner et al., 1995]. Furthermore, microbially produced surfactants offer several advantages over their chemical counterparts, owing to their ecological acceptance and lower critical micelle concentration (CMC) that offers more efficiency [Benincasa et al., 2002]. Currently, the main bottleneck for the widespread use of biosurfactants is the disadvantageous economics of their production. However, recently it has been shown that low-cost hydrophobic substrates, such as vegetable oils, can be used to produce rhamnolipids from *Pseudomonas aeruginosa*, which are among the most effective biosurfactants [Nitschke et al., 2005].

The fermentation process with *P. aeruginosa* (DSM 7108) to produce rhamnolipids using vegetable oils as carbon source has been developed at the Institute of Technical Biology, Technical University Karlsruhe, Germany. Using vegetable oil as the sole carbon source, about 88 g/l of rhamnolipids can be produced along with 75

g/l of wet biomass during a 260 hours long fermentation. 250 g/l of oil is fed during the process, out of which around 100 g/l is left unused in the broth. Initially, the biomass grows until 60 hours during which no significant rhamnolipids production takes place. Later, the cells reach the stationary phase with a biomass density of approximately 75 g/l (wcw) and rhamnolipids are secreted continuously into the broth. After around 260 hours, no further significant production of rhamnolipids is observed accompanied with a slight decrease in biomass concentration. The process is terminated and the batch is harvested. Biomass is separated by centrifugation and the supernatant is taken for product recovery.

As has been previously established that free surfactants in the medium could be toxic to cells [Schmid et al., 1998], the aim of the study was to separate the produced surfactant continuously (or semi-continuously) from the broth in order to allow more production. The applied methodology was to retain the resting cells in the bioreactor while drawing out the produced rhamnolipids along with unused oil. This was planned to be achieved by integrating a ceramic membrane into the bioreactor. The rationale behind our approach was based on the notion that addition of fresh oily substrate would ensure further production of rhamnolipids, while product inhibition would be checked by removal of the product from the culture broth.

5.2 Preliminary tests and membrane-module design

Based on the analysis previously carried out with yeast (*P. pastoris*, chapter 4), it was decided to develop submerged membrane system for retention of bacterial cells and recovery of product. The permeate thus contains rhamnolipids and oil along with the aqueous medium and salts. The pH of the process is maintained at 6.8 using H_3PO_4 (acid) and NaOH (alkali), and it has already been shown (chapter 3) that ceramic membranes are stable in this pH regime as well as against the chemicals used.

P. aeruginosa cells (employed in this study) are rod shaped and on average 1.8 μm in length and about 0.6 μm in width. The zeta potential of *aeruginosa* cells as well as rhamnolipids is in the same range (-20 to -40 mV) around the process pH [Sartor, 2006]. Monomers of rhamnolipid are about 0.1 μm long while the micelles are about 1 μm in diameter. However, micelles are reversible structures and break into

monomers under turbulent conditions as well as at higher pH [Oezdemir et al., 2004; Sanchez et al., 2007]. Thus, the separation in our case is solely based on size. Membranes with average pore size less than 0.4 μm were chosen for the investigation. Unlike in case of yeast filtration, monochannel membranes (with their selective layer on the inner side) were not the preferred choice, as the support layer which is about 0.6 - 0.8 μm wide could easily be fouled by bacterial cells. Hence, flat-sheet membrane with average pore size 0.2 μm (ItN Nanovation AG, Germany) was employed at first. The same was also used for feeding oil as emulsion and is discussed in chapter 6.

It was imperative to check the permeability of the desired product to check if there was any retention or adsorption on the membrane surface or inside the membrane matrix. To this end, aqueous rhamnolipids solution (JBR425) was obtained from Jensil Biosurfactant Company, USA and the vegetable oil was bought from the supermarket. Filtration tests were made with concentration of rhamnolipids ranging from 2 to 40 g/l, with and without 100 g/l oil in water. The amount of surfactants in feed and permeate was analyzed using 'Dr. Lange Schnelltest für Tensides' (chapter 3). Although this method is not very accurate for the determination of surfactant concentration as compared to HPLC, still it can provide a quick and fair comparison between the two set of samples. Due to hydrophilic character of ceramic membranes, oil permeation under pressure was not supposed to be a problem and the same was observed in these tests. The tests did not show any significant loss of surfactant in the permeate due to adsorption or retention by the membrane.

The stainless steel casing for the flat-sheet membrane was also modified by replacing the rectangular-box casing with the new cylindrical casing (fig. 5.1, left). The new casing is more advantageous in terms of stability against the shear stresses in the continuously stirred reaction volume. Furthermore, due to its geometry it should ensure smooth flow of the culture medium and less accumulation of the biomass over the module surface. Monochannel membrane with average pore size of 0.3 μm (with selective layer on the inner side) was also tested simultaneously under similar conditions (fig. 5.1, right) for comparison purposes.

Fig. 5.1: Cylindrical casing for flat-sheet membranes: an improvement over the rectangular ones (left); multichannel flat-sheet and monochannel cylindrical membrane integrated within the bioreactor (right).

5.3 Filtration of *P. aeruginosa* culture

Both the membrane modules were fitted as shown in figure 5.1 and the fermentation was carried out in the conventional mode as described above (section 5.1). It could be of interest that the fitted modules also act as baffles in the bioreactor and increase turbulence and hence enhance mixing in the medium. However, this effect has not been not been quantified in this study. After 200 hours of process time, filtration was started through the integrated membranes. The rhamnolipids and the oil concentrations in the culture at that time were about 70 g/l and 120 g/l, respectively. The biomass was at its highest level (75 g/l wcw) for the given system and the cells were in stationary phase. No flux was observed at all through either of the membranes. As a peristaltic pump was employed the suction pressure was low, which could otherwise have led to fouling due to cake layer formation on the membrane surface. Back flush was tried with fresh medium as well as with pressurized air but was not sufficient enough to clean the fouled membranes. Surprisingly, when the membranes were recovered after autoclave, no significant cell layer was found over the membrane surface (fig. 5.2). The membrane surface could be regenerated with simple cleaning under running water. However, the membranes were so severely fouled that it was not possible even to push compressed air through them (until 4 bar, the mean pore flow pressure was 3.12 bars). This led us to the conclusion that the fouling was inside the membrane matrix and not on the surface.

Fig. 5.2: Irreversible biofouling, no significant cell layer could be seen on the membrane surface and the surface could be regenerated by simple washing under water.

Complete blockage of the monochannel cylindrical membrane despite no cell layer on the surface could be understood as the support matrix is porous enough (0.6–0.8 μm) for the bacterial cells to penetrate. However, fouling of 0.2 μm flat-sheet membrane without any significant cell adhesion over the surface and inability to be cleaned by back flush showed that the inner matrix had been severely fouled by other components in the broth. Hence, it was a case of 'irreversible biofouling'.

5.4 Biofouling

Biofouling mechanisms include the adsorption of soluble and suspended components of the broth on membrane surfaces and in membrane pore, the clogging of membrane pore structure by fine colloidal particles and cell debris, and the adhesion and deposition of sludge cake on membrane surface [Liao et al., 2004]. Theoretically, there is defined a term 'critical flux' which means some threshold flux below which fouling does not take place. However, practically, this phenomenon is inappropriate to MBRs. This is due to the presence of colloidal and/or dissolved material which seems to deposit onto or within the membrane at all fluxes higher than zero [Judd, 2005]. Infact, it has been reported that there is fouling due to adsorption even at zero flux [McDonogh et al., 1992]. Biofouling results in reduced performances, severe flux decline, high energy consumption and frequent membrane cleaning or replacement [Judd, 2004].

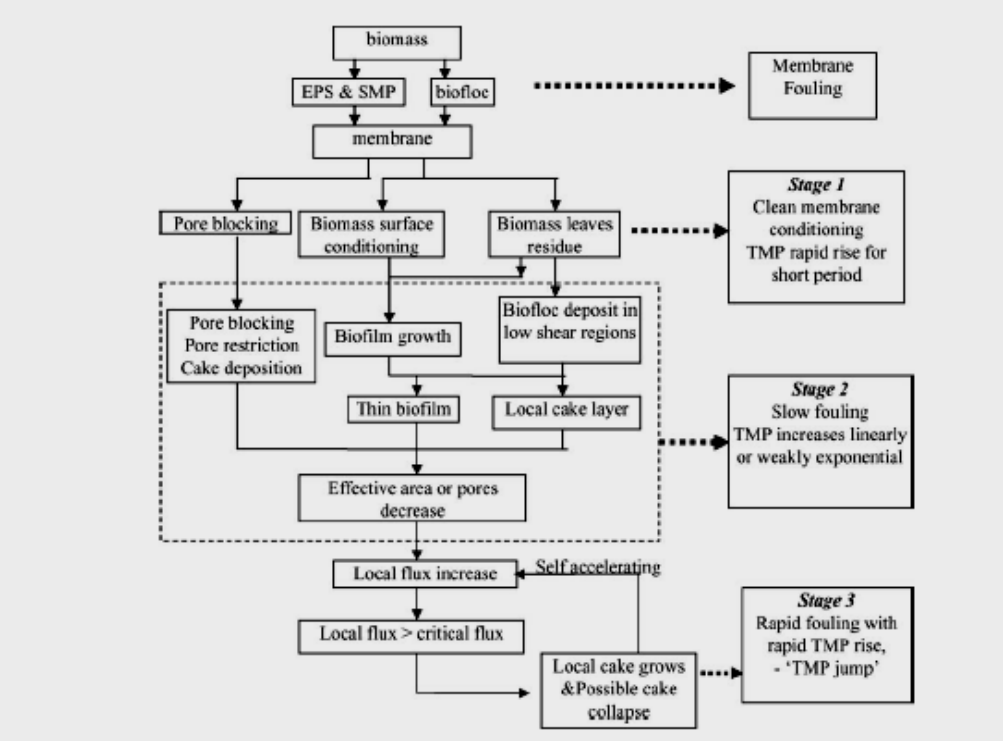

Fig. 5.3: MBR fouling mechanism map [Zhang et al., 2006].

5.4.1 Characterization of biofouling

Biofouling can be characterized on the basis of three fouling patterns: adsorption of EPS to the membrane surface, pore clogging by cells and cake or film formation arising from the deposition of cells or aggregates [Liao et al., 2004]. In the resistance-in-series model the filtration flux (*J*) is, at any stage, predicted using Darcy's law:

$$J = \Delta P / \eta . R_t$$

where ΔP is the pressure driving force (the TMP), η is the dynamic viscosity of the filter solution and R_t is the sum of the flow resistance [Kylloenen et al., 2005]. The total resistance is a function of the membrane resistance (R_m); the reversible resistance (R_r) which can be controlled with periodic breaks, back-washing as well as by scouring of membrane surface by air [Bouhabila et al., 2001; Germain et al., 2005]; and the fouling resistance, an irreversibly absorbed layer, which can not be removed by water washing (R_f) [Ognier et al., 2002].

$$R_t = R_m + R_r + R_f$$

In most cases, the cake layer on the membrane surface contributes to the reversible resistance, whereas pore clogging and mainly the adsorption of EPS onto the

membrane surface contribute to the irreversible fouling [Wang et al., 2007]. Various mathematical models have been reported for this effect in different filtration cases [Davies et al., 2000; Ognier et al., 2002; Li and Wang, 2006].

5.4.2 Extracellular polymeric substances (EPS)

In most environments, microorganisms (especially bacteria) grow in two distinct forms: as sessile cells within a biofilm attached to an interface or surface and as planktonik organisms freely dispersed in the aqueous phase. In most cases they prefer growing as sessile communities which is likely due to the protective nature of biofilm growth. Microorganisms growing within biofilm are more resistant to antimicrobial and biocidal agents, as the biofilm may prevent the biocide from reaching the inner-most cells by the presence of exopolysaccharide matrix [Walker et al., 2000].

Microbial extracellular polymeric substances are biosynthetic polymers (biopolymers). EPS are a complex mixture of proteins, carbohydrate, acid, polysaccharides, DNA, lipids and humic substances that surround cells and form matrix of microbial flocs and films [Wingender et al., 1999]. These EPS cause increase in the viscosity of the filter mixture and increase in filtration resistance of the membrane [Nagaoka et al., 1996]. EPS level has been identified as being primarily responsible for fouling in MBRs, representing up to 90% of the total filtration resistance [Clech et al., 2003; Nuengjamnong et al., 2005]. Mathematical models have been presented for biofouling by EPS in activated sludge systems by Nagaoka et al., 1998, in particular and by Kim et al., 2006, in general. Alginate belongs to the best studied bacterial extracellular polysaccharides and has often been used as a model compound in the study of the physiochemical and biological properties of EPS [Negaresh et al., 2006].

5.5 Simulated tests with *P. fluorescens* and alginate

Next, simulated tests were performed at the host laboratory in Saarbruecken in order to understand the fouling mechanism. Because of our limitations in working with GRAS organisms, experiments were conducted with cultures of *Pseudomonas fluorescens* instead of *Pseudomonas aeruginosa*. Average *P. fluorescens* cells are also 0.6 μm wide and 1.8 μm long just like average *P. aeruginosa* cells. Moreover, *P.*

fluorescens are also gram-negative and rod shaped, hence morphologically very similar to *P. aeruginosa* [Haas and Keel, 2003]. It is also known from literature that the main component of bacterial EPS is alginate [Negaresh et al., 2006]. A calculated amount of sodium salts of alginic acid (sodium alginate) was added to the reaction mixture in order to attain similar conditions of viscosity and fouling, as observed in the actual *aeruginosa* culture in the laboratory in Karlsruhe.

To this end, cylindrical monochannel membranes (ID=8mm, OD=14mm) with their thin decisive layer on the outer side were made available from atech innovations GmbH, Germany. The average pore size of the selected membrane was 0.3 µm. Cylindrical modules prepared for this study had a filtration area of approx. 22 cm^2.

5.5.1 Filtration with submerged membranes

Filtration set-up was established in a glass vessel equipped with an overhead stirrer. Submerged membrane module was connected to a constant-flux pump via pressure sensor and the TMP data was recorded in an attached computer. Overnight culture of *P. fluorescens* in LB medium with a total reaction volume of 500 ml was used. The mixture had: biomass 40 g/l (wcw), oil 100 g/l, rhamnolipids 40 g/l and alginate 0.02% (w/v). The viscosity of the mixture was around 6-8 cSt which was approximately double than that of actual *aeruginosa* culture producing rhamnolipids. As in the actual culture apart from alginate other EPS are also present (which have not been characterized in this study). Thus, it was assumed safe to investigate with approximately double viscosity while taking alginate as the only EPS. The constant-flux pump was set to obtain a flux of 28 l/(m^2.h) through the membrane and the TMP was observed to analyze the resistance to flow.

As shown in figure 5.4 the transmembrane pressure kept increasing throughout the test duration. As expected, there was a quick rise in TMP in the beginning because of the ‘conditioning’ of membrane surface with cells. Later, the TMP rise was gradual but continuous. Unlike in the case of filtration of yeast culture (chapter 4) a constant value reach in TMP was not observed here. After one hour, the membrane module was taken out from the mixture to check the state of fouling. The used membrane was compared with a similar unused one (fig. 5.5).

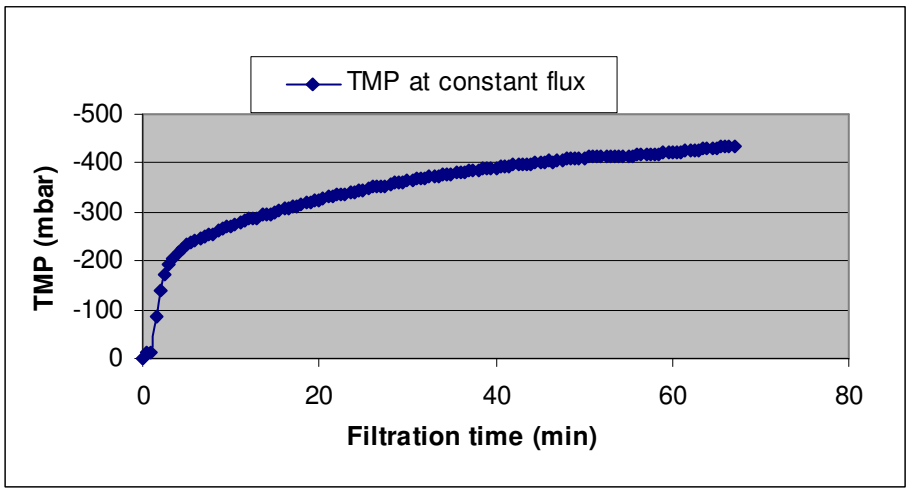

Fig. 5.4: TMP data for the submerged membrane in culture mixture at a constant flux of 28 l/(m^2.h).

Fig. 5.5: Visual comparison of membrane for biofouling: membrane after one hour filtration (right), unused membrane (left).

Fig. 5.6: Comparison of filtrate and culture mixture, the eppendorf tubes (after centrifugation) are placed aside their respective solutions.

As could be observed in the previous figure, the used membrane showed a very thin cell layer on the surface. This result was more significant than what was observed in the experiment conducted in Karlsruhe with *aeruginosa* culture. However, the membrane surface could still be regenerated in a similar manner. The permeate was clear (fig. 5.6) and was checked for presence of cells by centrifugation method. As can be seen in figure 5.6, the eppendorf tube containing culture mixture had a thick cell palate at the bottom after being centrifuged at 4500g force for 4 minutes. No such palate was observed with the permeate and the absence of cells was also confirmed by the agar-plate test.

The amount of rhamnolipids in the permeate was checked at regular intervals. No significant difference with concentration in the culture mixture was observed. Similar observation was made with respect to the oil concentration. However, continuous rise in TMP showed that there was fouling of the membrane matrix with increasing filtration time. This was confirmed by comparing the pure water flux of unused and used membranes. The pure water flux of the membrane decreased from 780 to 132 l/(m^2.h.bar) after being used for filtration of the culture mixture (fig. 5.9).

5.5.2 Adsorption of alginate in the membrane

For the above test, alginate was added as the only EPS in the test solution. Hence, it was important to check its permeation through the membrane. The concentration of alginate was checked at regular intervals in permeate as well as in the feed solution. Though the flux was constant, the sieving coefficient for alginate decreased to approximately 11% during one hour filtration (fig. 5.8). The sieving coefficient is defined as the ratio of the amount of filtrate compound (alginate in this case) in permeate and in the feed [Harscoat et al., 1999]. Moreover, the concentration of alginate in the feed solution increased a little (approximately 1.5%) during that time. Taking into account the volume of permeate collected and the amount of alginate in the permeate, the concentration of alginate in the feed should have increased more. It showed that only a little amount of alginate was retained by the membrane at the surface; most of it was adsorbed in the membrane matrix. This phenomenon of adsorption of alginate in ceramic membranes has been shown as well in earlier studies [Cheze-Lange et al., 2002].

To analyze the effect of alginate in membrane fouling it was decided to repeat the filtration test under the same conditions but with alginate-only solution. This would also quantify the fouling of membrane due to alginate and therefore give a fair comparison with overall fouling during filtration of culture mixture.

As shown in figure 5.7, the TMP for the filtration of alginate solution rose in a similar manner as observed in figure 5.4 for the filtration of the culture mixture. However, the value attained was different for a given flux and filtration time. The TMP value after one hour of filtration was approximately 64% of the value observed for the filtration of

the culture mixture. Thus, it is concluded that there are other components responsible for membrane fouling and hence the rise in TMP. But as expected, alginate contributes the maximum to this effect. More interestingly, almost a similar decrement in the sieving coefficient of alginate was observed in both the cases (fig. 5.8).

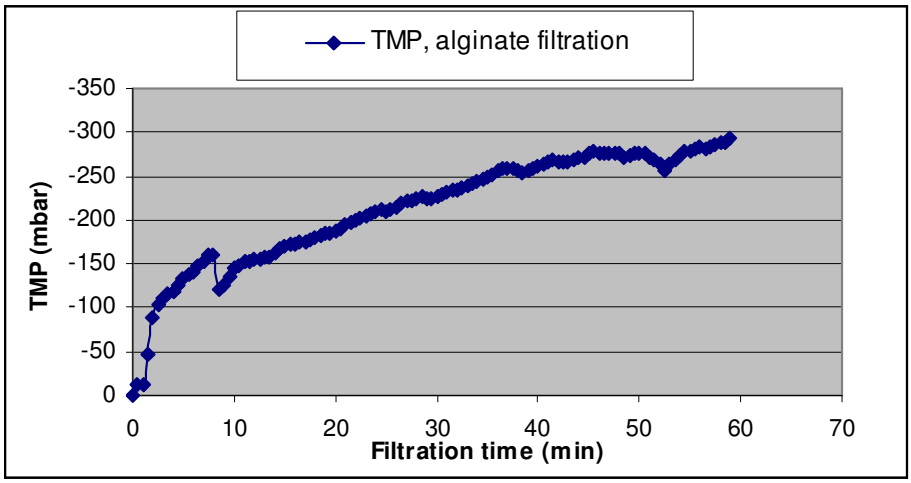

Fig. 5.7: TMP data for filtration of alginate solution at a constant flux of 28 l/(m^2.h).

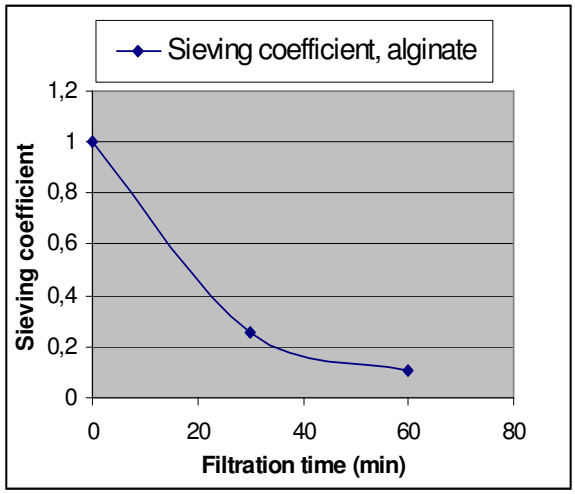

Fig. 5.8: Sieving coefficient of alginate through the ceramic membrane, decrease is shown during one hour filtration time.

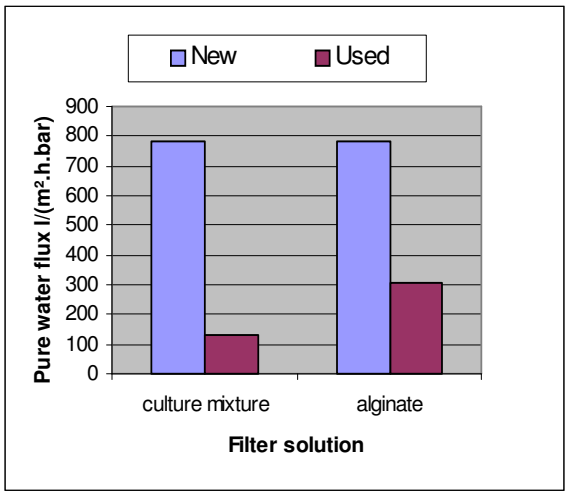

Fig. 5.9: Comparison of pure water flux, for the membrane used for filtration of culture mixture and alginate solution respectively.

The molecular weight of alginate as reported by the manufacturer lies between 48 to 186 KDa. This is due to polymerizing tendency of the monomers and could indeed be a reason for fouling of the membrane matrix. Also, the hydrophilic nature of ceramic membranes would favor the adsorption of alginate [George and Abraham, 2006].

Fouling due to alginate was also confirmed by comparing the pure water flux of unused and used membrane. The flux reduced from 780 to 304 l/(m^2.h.bar) after filtration of alginate solution. The difference in reduction of water flux of used membranes in the two tests (fig. 5.9) again confirmed that other components, which could not be characterized in this study, were also responsible for membrane fouling, although it is to be noted that most of the fouling of the membrane matrix was due to alginate.

5.5.3 Biofilm formation during growth phase?

In the simulated tests a thin but a bit clearer cell layer was observed on the membrane surface after filtration (fig. 5.5), as compared to the layer observed with the actual culture. The concentration of rhamnolipids as well as of oil was in the same range as in the actual culture. However, no fluxes were observed with the actual culture, while in simulated tests clear permeate was collected and fouling was also observed, mainly due to alginate as discussed above. These differences could be attributed to the fact that in the simulated tests overnight grown culture was used, while in actual test the membrane was already submerged in the culture during the growth phase of cells.

To test the possibility of membrane fouling by cells during growth phase a similar membrane module was left in the overnight culture of *P. fluorescens*. No significant cell layer was observed at the surface after 16 hours of inspection. It confirmed that the cell layer formation occurred due to suction pressure during filtration. The module was washed under water and then checked for permeability. The membrane was completely fouled and it was not possible to push even pressurized air through it, even when the membrane was completely dried under vacuum and gassing was tried until 4 bars (the mean pore flow pressure for the membrane was 2.38 bars) of the membrane. This corroborates the observation earlier made with the *aeruginosa* culture in Karlsruhe.

It might be a possibility that during growth phase, when the cells are small enough to penetrate through the membrane pores, they flow into the submerged membrane along with circulating culture solution. Inside the matrix they find a protective shelter to avoid continuous shear due to turbulence in the circulating fluid. Thus, they are

able to form a biofilm at the porous and rough surface inside the unsymmetrical matrix while an energy source is available from the flowing medium. Later the membrane is severely fouled making it unfeasible to achieve any flux at all. The test with membrane left in the overnight culture showed a high tendency of *aeruginosa* cultures to form biofilms when appropriate surfaces are available. This might be the reason that at least a little success has been reported for filtration of *aeruginosa* culture to recover rhamnolipids with external cross-flow systems, even though membrane fouling occurred eventually [Gruber, 1991].

5.6 Control of biofouling

The reported methods/recommendations to control biofouling during filtration of broth include:

- optimal hydrodynamic conditions and membrane module design;
- use of negatively charged membrane;
- periodic backwash with permeate or fresh substrate;
- periodic breaks in permeability to enhance shear forces on the surface;
- and chemical cleaning [Liao et.al., 2004].

For the current study these options are either not applicable or have not been useful (section 5.3). The ceramic membranes used in this study have negative charge in the pH range of the bioprocess. The hydrodynamic conditions inside the reactor as well as the geometry of membrane module are sufficient to avoid any significant cell adhesion on the membrane surface, and the same has been observed in this study. Applied suction pressure was not high at all. Backwashes as well as periodic breaks have not been useful. As the membrane is submerged in the reaction medium, chemical cleaning is not feasible. Thus, some other strategy is needed to control biofouling in this system.

5.6.1 Arrangement of membrane in bioreactor

As it was observed that biofilm formation takes place mainly during the growth phase of cells, one strategy to avoid membrane fouling due to biofilm formation could be to immerse the membrane into the culture at a later stage. As discussed earlier, there is no significant rhamnolipids production in the growth phase, and in the production

phase the cells are stationary. The method is schematically depicted in figure 5.10. In this way, filtration could be achieved like in the case with overnight culture. However, fouling due to EPS could not be checked.

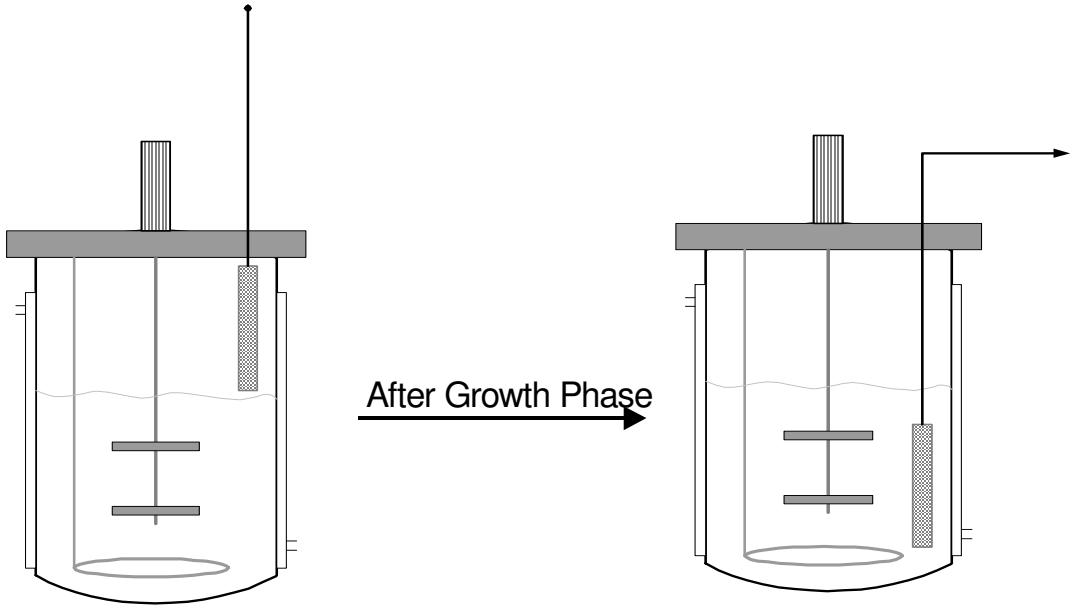

Fig. 5.10: Adjustable arrangement of membrane in bioreactor to avoid biofouling due to biofilm formation during growth phase of cells.

Nevertheless, the method needs to be tested and compared with earlier results. At least it could be useful with bacterial cultures which do not produce any high amounts of EPS. Moreover, for yeast fermentation, membranes with bigger pores could be used. This would significantly enhance the flux while avoiding any accumulation of growing cells inside the membrane matrix.

5.6.2 Polymer coating on ceramic support

Earlier studies with ceramic membrane employed for polysaccharides recovery from fermentation broth have always reported irreversible fouling due to adsorption of polymers [Harscoat et al., 1999; Cheze-Lange et al., 2002]. The superficial layer created by cells on the membrane surface can be removed easily by washing that has also been shown in the study above. Furthermore, alginate is hydrophilic in nature [George and Abraham, 2006] and hence has more affinity for hydrophilic ceramic surfaces, leading to extended fouling.

One way to minimize the adsorption of hydrophilic polysaccharides in the membrane matrix could be the reduction of the hydrophilic character of the membrane. This could be achieved by coating a thin layer of hydrophobic polymer such as PPG inside the unsymmetrical matrix. Thus, maintaining the mechanical and thermal strength as well as higher flux characteristics, while altering the membrane selectivity. This is discussed in detail in chapter 7. However, fouling due to other hydrophobic components in the broth such as proteins could not be neglected.

5.6.3 Ultrasound therapy against biofouling

Ultrasonic techniques provide an alternative method for membrane fouling control and membrane cleaning. In a liquid medium, ultrasound creates regions of high and low pressures. Acoustic streaming, microstreaming, microstreamers, microjets and shock waves are generated as a result of ultrasound [Kylloenen et al., 2005]. These processes result in fluid movement and have been shown capable of removing portions of the foulant layer from the membrane surface, and preventing the deposition of particles that lead to membrane fouling [Chen et al., 2006A]. In addition, ceramic surfaces are known as good reflectors of ultrasonic waves and hence offer better response when subjected to ultrasonic field [Chen et al., 2006B].

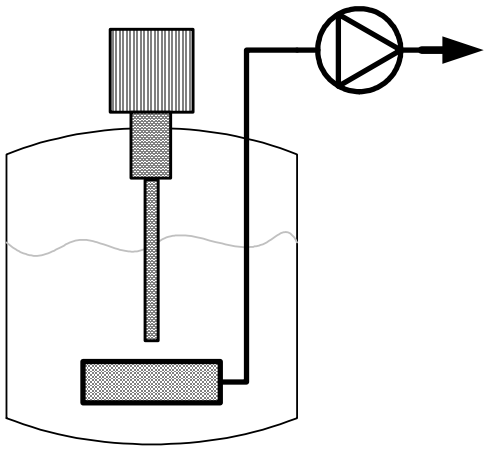

Fig. 5.11: Submerged membrane placed under ultrasonic homogenizer.

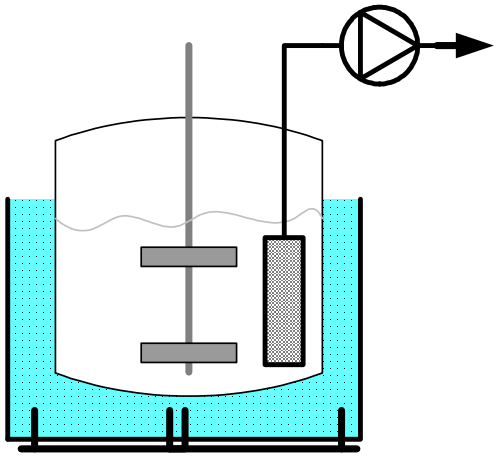

Fig. 5.12: Membrane submerged in culture placed in ultrasonic bath.

Two sets of ultrasound tests were performed. In the first set the submerged membrane was placed under the ultrasonic homogenizer (SONOPLUS HD2070,

Bandelin electronic GmbH, Germany) fitted into the glass reactor from the top (fig. 5.11). The mixing was achieved with magnetic stirring from the bottom. The working frequency of the homogenizer was set to 20 KHz with an adjustable power input. In the second set (fig. 5.12) the whole glass vessel, including the stirrer and the submerged membrane, was placed in the ultrasonic bath (SONOREX RK100H, Bandelin electronic GmbH, Germany). The ultrasonic bath was operated at 35 kHz and at fixed power. As discussed before (section 5.5.1), same culture mixture and membrane were used and the effect was analyzed in terms of TMP required for the same flux, but in presence of ultrasonic effect.

In both the cases, the value of TMP reached approximately -260 mbar after one hour of filtration while a flux of 28 l/(m^2.h) was obtained, which was almost half as was observed without ultrasound. However with the membrane placed under homogenizer, the permeate was not clean even after 30 minutes of filtration. A lot of cell debris was observed. When tested on agar plate, a significantly high number of colonies were observed. The membrane was washed and characterized again using flow-pore method. It was noted that the average pore size had not changed significantly but the pore distribution showed that the number of smaller pores were less as compared to the unused membrane. This led us to conclude that the surface portion of membrane directly under the homogenizer was the most affected.

On the other hand, the permeate from the second set was also not clear. None the less, only a few colonies were observed when the sample was cultured on agar plate. No significant alteration in the average pore size and pore distribution was observed for the employed membrane. Nevertheless, in both the cases significant cell death occurred and the retention was not complete. However, the membrane was affected more in the first case. Permeate from both the sets was also checked for loss of membrane material during sonification. Samples were analyzed using atomic adsorption spectrometry (AAS) for an amount of Al atoms and the corresponding amount of Al_2O_3 was calculated. The amount of Al_2O_3 in samples was 38 and 30 µg/l respectively and hence no significant loss was observed from the membranes during the tests. However, significant loss of ceramic material from membrane during longer exposure to ultrasound can not be ruled out [Chen et al., 2006A].

5.7 Recovery of polyhydroxyalkanoates from *P. aeruginosa* culture

The process for production of polyhydroxyalkanotaes (PHA) with *Pseudomonas aeruginosa* 42A2 (NCBIM 40045) using waste oils as carbon source has been developed at Department of Microbiology and Parasitology, University of Barcelona, Spain [Guerrero et al., 1997]. Owing to their unique polymerization characteristics and biodegradability, PHAs have vital applications in biopolymer industry [Braunegg et al., 1998]. The aim of this study was to recover the secreted polyhydroxyalkanotes (PHAs), a mixture of mono-, di- and tri-hydroxyalkanotes, semi-continuously from the broth in order to avoid high accumulation which leads to further degradation of the product by various extracellular enzymes [Cullere et al, 2001].

As has been already observed (and discussed above), biofouling of the membrane with extracellular polymeric substances (EPS) is the major limitation in employing them for product recovery from *aeruginosa* culture. Moreover, it has been observed (section 5.5.3) that *P. aeruginosa* cells have high tendency to form biofilms inside the membrane matrix during the growth phase. To counter this effect it was decided to introduce the ceramic membrane into the culture solution when the cells are in stationary phase. The membrane was placed in the headspace (fig. 5.11) above the antifoam disk (as a lot of foam is produced during the process) and was immersed in the culture solution when the cells were in stationary phase. One more membrane was placed between the impellers of the bioreactor and was used mainly for feeding the oily substrate into the medium that is discussed in chapter 6. Both the membranes were of average pore size 0.4 μm, had cylindrical geometry (ID=8mm, OD=14mm) and had their thin decisive layer on the outer surface (ItN Nanovation AG, Germany). The available filtration area was approximately 22 cm^2.

After 60 hours of the process time, filtration was tried with the membrane placed between the impellers and as expected, no significant flux could be achieved even at 2 bars of applied transmembrane pressure. However as the same membrane was used in this process for feeding the substrate, some oil (which might have remained in the tubing) was recovered during the initial suction. The last feeding was performed at 43 hours of process time and the TMP values did not show any significant obstruction to the flow of oil through the membrane. This showed that

regular back flush could control the membrane fouling. Next, the membrane that was placed in the headspace was immersed into the culture solution. Suction was performed using the peristaltic pump fitted with the bioreactor (Biostat B2, B. Braun GmbH, Germany) at a constant TMP of 0.5 bars. A flux of approximately 36 $l/m^2.h$ was achieved in the beginning, which fell down to zero within 20 minutes of filtration. The TMP was raised stepwise up to two bars but no more flux could be obtained showing complete fouling of the membrane with extracellular components.

Fig. 5.13: Adjustable arrangement of integrated membranes, monochannel ceramic membranes integrated with in the bench scale bioreactor for product recovery. The first membrane was placed between the impellers to provide maximum turbulence over the membrane surface. The second membrane was placed in the head space above the antifoam disk (left) and was immersed into the culture (right) when the cells were in stationary phase.

The permeate collected was not clear but did not show any cell pallets after centrifugation. No loss of product due to adsorption on the surface or retention in the membrane matrix was observed. However, the fouling of the membrane with the culture components once again denied the application of submerged ceramic membrane systems for product recovery from *aeruginosa* cultures.

5.8 Recovery of lysine from *C. glutamicum* culture

To reiterate, so far in this chapter, filtrations with *P. aeruginosa* and *P. fluorescens* have been reported. However, as *Pseudomonas* are very well known for their biofilm forming tendency [Walker et al., 2000], it became imperative to investigate the

filtration with a different bacterial culture which offers different surface properties as compared to *Pseudomonas* (so preferably not known for biofilm formation), but is well known and could be commercially used. *Corynebacterium glutamicum* is an industrial microorganism which has been widely studied, characterized and developed for the production of a range of industrial products such as acetate, pyruvate, ethanol, D- and L-lactate, succinate, L-lysine and L-serine, etc. [Wendisch et al., 2006]. L-lysine is an essential amino acids required for nutrition of animals and humans. *C. glutamicum* is the most important organism for industrial lysine production, and the only other species used for lysine production being recombinant *E. coli* [Wittmann and Becker, 2007]. However, *E. coli* is well known for its biofilm forming capabilities [Walker et al., 2000]. Hence a model investigation was carried out only with wild type culture of *C. glutamicum* for recovery of lysine via submerged ceramic membranes.

For these tests the wild type culture of *C. glutamicum* (ATCC13032) was generously provided by the research group at Department of Technical Biochemistry, University of Saarland, Saarbruecken, Germany. The preculture and the main culture was obtained in complex medium and minimal medium respectively to ensure that the culture itself does not produce any lysine (as suggested by the research group at the Technical Biochemistry Department). Lysine was added to the overnight cultures and filtrations were carried out in a similar way as was done with *P. fluorescens* culture (section 6.5). The ceramic monochannel membrane employed had an average pore size of 300 nm (ItN Nanovation AG, Germany) and a filtration area of approximately 22 cm^2. The amount of lysine in permeate was analyzed with HPLC. To check the biofilm formation during growth phase, as has been previously observed with *P. aeruginosa* and *P. fluorescens* cultures, one of the membrane was left in the overnight culture. After 16 hours of inspection time, the membrane showed a little fouling that could be cleaned by back flushing with water at 1.5 bars TMP. This fouling was attributed to the accumulation of growing cells inside the membrane matrix and hence biofilm formation could be completely ruled out.

Next, filtration was performed with lysine added to the overnight culture. The highest conversion of glucose to lysine with *C. glutamicum* achieved in practice is in the range of 55% (mol/mol) [Wittmann and Becker, 2007]. As the minimal medium

contained 25 g/l glucose, 10 g/l of lysine was added to the filtration mixture. The biomass concentration in the filter solution was about 55 g/l (wcw). The mixing was set at 500 rpm and fresh membranes were introduced into the culture mixtures. Prior to this, filtration of lysine-only solution was performed with similar membrane and no significant difference was observed in lysine concentration in the feed and in the permeate, thus denying any loss of lysine due to adsorption on the membrane surface or inside the membrane matrix. The constant-flux pump was set to obtain a flux of 56 l/(m^2.h) through the membrane and the TMP was observed to analyze resistance to flow.

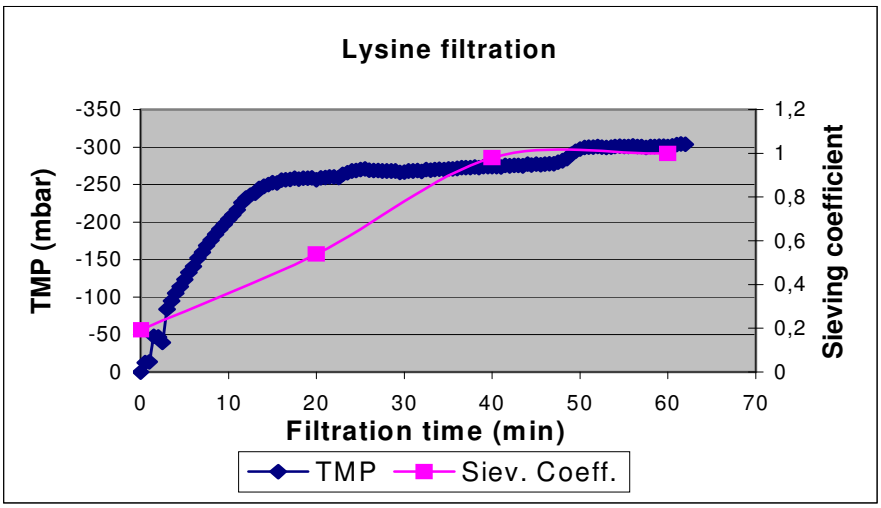

Fig. 5.14: Recovery of lysine from *C. glutamicum* culture: the TMP for a flux of 56 l/(m^2.h) as well as the sieving coefficient for lysine are shown during the filtration via submerged membrane.

It is to be noted that filtration was performed at a flux of 56 l/(m^2.h) which was twice the value set for *P. fluorescens* filtration (section 5.5). As expected, the TMP increased rapidly (fig 5.14) in the beginning due to conditioning of the membrane surface by cells. The sieving coefficient for lysine was low in the beginning but a stable, maximum value of 1 was attained after 40 minutes of filtration. Thus, there was no loss of product due to adsorption in the membrane matrix, and the low sieving in the beginning could be attributed to the formation of cell layer on the membrane surface which might have absorbed some of the lysine. After 60 minutes of filtration, the membrane back wash was performed with water and a clean membrane could be regenerated. No significant difference in TMP was observed when the regenerated membrane was used for filtration of a similar mixture. Hence, the filtration of *C.*

glutamicum for recovery of lysine using submerged ceramic membrane system was successfully established.

5.9 Conclusion and outlook

Filtrations of bacterial culture (*Pseudomonas sp.*) with microporous-submerged-ceramic membranes have been investigated. The filtrations did not show satisfactory results due to severe fouling of the membrane matrix with the extracellular polymeric substances (EPS), mainly alginate, produced by *Pseudomonas aeruginosa* cells.

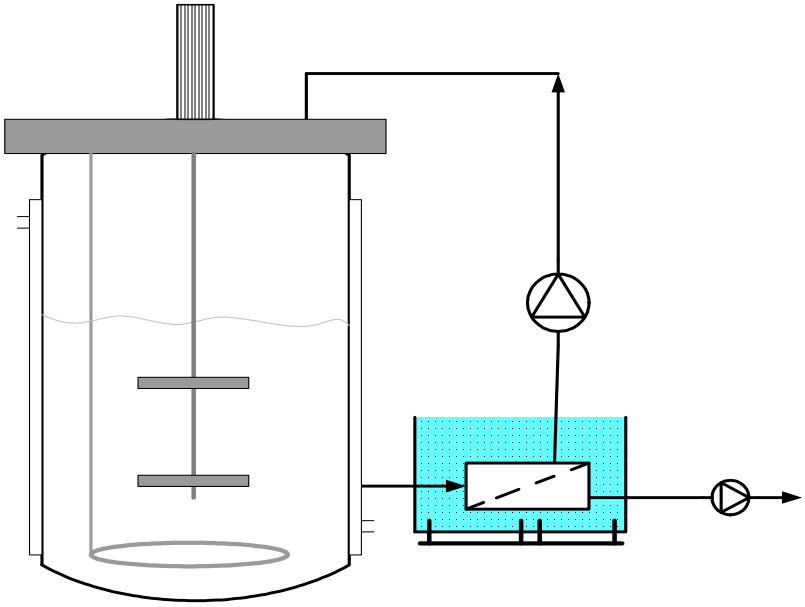

Fig. 5.15: Ultrasound therapy against biofouling: bioreactor integrated external cross-flow membrane system submerged in an ultrasonic bath.

The employment of ultrasound showed a positive effect on filtration in terms of reduced TMP for the same flux through the submerged membrane. However, cell death remains a major issue when applying ultrasound as a fouling control measure. One way to minimize cell death could be the use of significantly lower frequencies and energy input. The aim should be to induce enough cavitation so that adhesion and/or deposition on the surface and agglomeration of the compounds (polysaccharides, proteins, etc.) could be avoided. Moreover, ultrasonic waves are less harmful for stationary cells [Vollmer et al., 1998]. One such integrated system could include an external cross-flow module submerged in ultrasonic bath (fig. 5.15).

The retention time of cells (and hence the cell death) in the ultrasonic field could be controlled by adjusting the flow velocity. This should also improve permeability by enhancing micelle breakage in case of surfactant recovery and by reducing the agglomeration in case of polysaccharides. Short burst of ultrasonic power could be used to avoid significant cell death and membrane material loss while saving energy at the same time. However, investigations with external membrane systems were beyond the range of this work.

Nevertheless, filtration of non-biofilm forming bacterial culture (*Corynebacterium glutamicum*) has been shown successfully. Thus, the submerged ceramic membrane system can be employed for recovery of low molecular weight products from high cell density bacterial cultures as well, which are neither forming biofilms nor producing a high amount of EPS.

6 Substrate feeding: Aqueous two-phase systems

6.1 Significance of aqueous two-phase systems

Two liquid phase bioprocesses where cells are cultivated in media consisting of aqueous solution and an organic/water-immiscible solvent, offer a valuable biotechnological tool for biotransformation of apolar compounds [Schmid et al., 1998]. The immiscible or apolar phase could be a substrate in itself, or a reservoir for substrate and/or the product, thus minimizing inhibition to growth and production by regulating the concentrations in the aqueous biocatalysis environment [Buehler et al., 2002]. The partitioning between the two phases is dependent on the surface properties, conformation of the materials, and on the composition of the two-phase system. The practical application of aqueous two-phase systems (ATPS) to bioprocess development has been exploited for several years for the recovery of biological products, and by proper design of the two-phase system it is possible to obtain the product in a cell-free stream [Rito-Palomares, 2004]. Its earliest use involved the *in situ* removal of inhibitory products, mainly ethanol, acetone, butanol and organic acids. Because of its simplicity in operation and high holding capacity, easy scalability and ability to hold high biomass load in comparison to other separation techniques, aqueous two-phase systems are well suited for large-scale purification of biomaterials such as enzymes and other specific proteins from crude feed-stocks. The concept of the two-phase partitioning bioreactor has also been applied to controlled delivery of a toxic substrate dissolved in an organic phase to a cell-containing aqueous phase [Malinowski, 2001]. The system is thus well suited for biodegradation of hazardous pollutants.

However, such systems are limited by high mass transfer rates due to low miscibility of the two phases. To improve these rates it is required to enhance the organic-aqueous interfacial area in the reaction system. This could be achieved by increasing the volume fraction of the dispersed organic phase either by increasing the agitator input or using surfactants [Schmid et al., 1998]. This in turn increases the shear forces in the system that might affect the metabolism of cells, and also demands high energy input which is significant when considering the scale-up of the bioprocess.

Surfactants help in enhancing the accessibility of the immiscible substrate by decreasing the surface tension of aqueous phase and hence improving the miscibility. However, their concentration in the medium, if not regulated, could also pose some negative effect on cell metabolism [Schmid et al., 1998]. Thus, a methodology needs to be developed to enhance the organic-aqueous interfacial area, and hence the mass transfer to microorganisms while keeping the energy input in check.

6.2 Emulsification using microporous system

6.2.1 Emulsions

Emulsions are dispersed systems of two (or more) insoluble liquids, for example water and oil. If oil droplets are dispersed in water/aqueous phase, the system is called oil-in-water emulsion (O/W). Vice versa it is called water-in-oil (W/O). Emulsions play an important role in the formulation of cosmetics, pharmaceuticals, paints and food. The most common examples from daily life are mayonnaise (O/W) and margarine (W/O). Emulsions are also encountered in the petroleum industry, especially during crude oil production, as well as in some solvent extraction processes [Lambrich and Schubert, 2005]. Depending on the emulsification process, the diameter of the droplets lies between 0.1 μm and 0.1 mm. Such emulsions are thermodynamically unstable, which means that they have a tendency to reduce the interface as a result of a relatively high interfacial tension. Coalescence, an irreversible process, leads to the formation of larger droplets due to the fusion of two or more smaller droplets [Charcosset et al., 2004].

Emulsions are usually prepared by using high-pressure homogenizers, ultrasound homogenizers and rotor systems such as colloid mills, stirred vessels or toothed disc dispersing machines. Turbulence is the primary cause of fluid disruption leading to the formation of droplets. Larger droplets are continuously disrupted by the shear force caused by the flow conditions in these devices. The energy consumption is usually very high. The main disadvantage of these methods is the high mechanical stress due to the forces in the flow field; pressures in the range 5.0×10^6 to 3.5×10^7 Pa are common [Lambrich and Schubert, 2005]. Therefore, shear sensitive

ingredients such as proteins or starches may loose functional properties. Moreover, the resulting droplet size distribution is significantly large and cannot be easily controlled. Reproducibility is often poor and the quality of the product can vary per batch on the same manufacturing scale. Thus, scale-up is a common difficulty [Graaf et al., 2005].

6.2.2 The membrane emulsification process

In case of membrane emulsification, droplets grow at the pore outlets until upon reaching a certain size, when they detach (fig. 6.1). This is determined by the balance between the drag force on the droplet from the flowing continuous phase, the buoyancy of the droplet, the interfacial tension forces and the driving pressure (TMP). The final droplet size and size distribution are determined by the pore size and size distribution of the membrane (which can be characterized beforehand) and also by the degree of coalescence, both at the membrane surface and in the bulk solution. The porosity of the membrane surface is also an important parameter for the emulsification process: it determines the distance between two adjacent pores. In a typical membrane emulsification system the phase to be dispersed is pumped under pressure through the membrane pores into the continuous phase which circulates tangentially to the membrane surface [Charcosset et al., 2004].

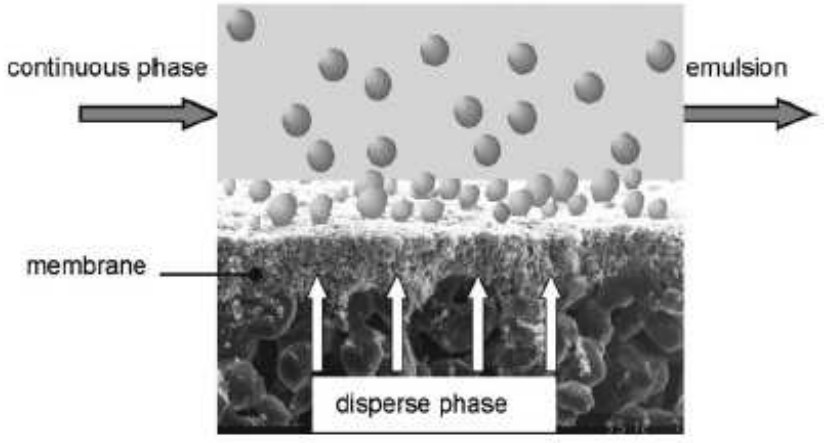

Fig. 6.1: Droplet formation at the membrane surface [Lambrich and Schubert, 2005].

Membrane emulsification has mainly been investigated and developed in the last 15 years. As compared to conventional methods, the distinguishing feature is that the resulting droplet size is controlled primarily by the choice of the membrane and not by

the generation of turbulent droplet break-up. Hence, the apparent shear stress is lower than the above mentioned conventional emulsification systems. Indeed, the droplets are directly formed by permeation of the dispersed phase through the micro-pores, instead of disruption of large droplets in zones of high energy density. Besides the possibility of using shear-sensitive ingredients, emulsions with narrow droplet size distribution can be produced. Furthermore, membrane emulsification processes allow the production of emulsions at lower energy input (10^4 – 10^6 Jm^{-3}) as compared to conventional mechanical methods (10^6 – 10^8 Jm^{-3}) [Lambrich and Schubert, 2005].

It has been recognized that the average droplet diameter $\mathbf{d_d}$ increases with the average membrane pore diameter $\mathbf{d_p}$ by a linear relationship, for a given operating condition:

$$\mathbf{d_d = c\, d_p}$$

where **c** is a constant for that particular membrane type. The maximum membrane porosity to prevent coalescence of droplets growing on neighboring pores is reported to be 1.5% [Charcosset et al., 2004].

The most commonly used membrane for the preparation of emulsion is Shirasu porous glass (SPG) membrane synthesized from $CaO\text{-}AL_2O_3\text{-}B_2O_3\text{-}SiO_2$ type glass, which is made from '*Shirasu*', a Japanese volcanic ash. In addition to SPG membrane, emulsions have been successfully prepared using commercial micro filtration membranes such as ceramic aluminum oxide ($\alpha\text{-}Al_2O_3$) membranes, zirconia coated membranes and polytetrafluoroethylene (PTFE) membranes [Charcosset et al., 2004].

The potential disadvantage of direct membrane emulsification is the relatively low maximum disperse phase flux through the membrane (typically 0.01 to 0.1 $m^3/ m^2.h$). This low flux is needed to avoid the transition from a 'size stable' to 'continuous outflow' zone and to avoid steric hindrance among droplets that might be formed simultaneously at the adjacent pores. To avoid this possible limitation, various operating methods have been introduced such as rotating membranes and repeated membrane extrusion of coarsely pre-emulsified feeds [Charcosset, 2006].

6.3 Biosurfactant production from vegetable oils

6.3.1 Background and objective

This study was aimed at developing a bioprocess for cost-effective production of biosurfactants (rhamnolipids). Biosurfactants are of industrial interest due to their broad range of applications including emulsification, phase separation, wetting, foaming, solubilization, emulsion stabilization, de-emulsification, corrosion inhibition and viscosity reduction [Ochsner et al., 1995]. Rhamnolipids are rhamnose-containing glycolipids having major application in detergents. Those produced by *Pseudomonas aeruginosa* have been widely studied [Jeong et al., 2004].

A possible strategy to reduce biosurfactant production costs is the use of inexpensive substrates such as agroindustrial wastes. The limiting factor is the solubility of oil in the aqueous media at moderate operating conditions, as high mixing/stirring could lead to high shear stress for the microorganism. Also, a homogenous dispersion of oil could enhance the oxygen solubility in the reaction mixture [Jia et al., 1996].

Emulsified feeding of the oily substrate into the aqueous medium was investigated to enhance the interfacial area by obtaining a homogeneously dispersed oil phase, and hence improve the mass transfer in the aqueous-organic mixture.

6.3.2 The conventional bioprocess

The fermentation process (process A) with *Pseudomonas aeruginosa* (DSM 7108) to produce rhamnolipids using vegetable oils as carbon source has been developed at the Institute of Technical Biology, Technical University Karlsruhe, Germany. A total of about 88 g/l of rhamnolipids are produced during 260 hours long bioprocess. The final biomass concentration is about 15 g/l dry-cell-weight which corresponds to 75 g/l of the wet-cell-weight. This is to be noted as the highest concentration possible with the bench scale system used for this study. In another fermentation with the same system but inoculated with frozen cells from the harvest of a previous fermentation, the biomass level kept at 75 g/l (wcw) throughout the process, which was the same as at the time of inoculation. A total of 250 g/l of oil is used, with 125 g/l added at the time of inoculation and the rest after 50 hours when the biomass in the reactor reaches about 50 g/l of the wet-cell-weight. The total reaction volume is 2 liter. The

rate of mixing is adjusted between 600-900 rpm due to employment of antifoam disc (fig. 6.2) in the head space of the bioreactor, as high amount of fine and very stable foam is produced during the bioprocess. However, such high rate of agitation would demand high power input when operating at higher scales. Hence, a method has to be developed to obtain the same level of mixing of the two phases but with lower energy requirement.

6.3.3 Emulsified feeding via membrane

To achieve direct emulsification in the reaction volume, membranes with their active or decisive thin layer on the outer side are required [Chmiel, 2004]. For the bench scale reactor available, it is better to use a thin cylindrical membrane, but as these type of membranes are normally produced to be used in cross-flow mode (with the thin decisive layer on the inner side), flat-sheet membranes of average pore size 0.2 µm (ItN Nanovation AG, Germany) fitted in new cylindrical casing (chapter 5, fig. 5.1) were used for the first experiment (process B). The standard (10 cm long) pieces were used and cut to a width of 3 cm so as to fit within the available space. The total area available to create fine emulsion in the aqueous medium was approximately 60 cm^2. The module was fitted against the flow inside the reactor so that the droplets should have swept away as soon as they were formed, with the medium flowing over the surface. This phenomenon is believed to be helpful in avoiding coalescence of the droplets coming out of the pores at the membrane surface.

Later, monochannel membranes with the active layer on the outer surface were made available from atech innovations GmbH, Germany. Modules were prepared so that the small cylindrical piece is placed between the two impellers inside the bioreactor. In this way, the oil droplets should have been swept away from the membrane surface as soon as they were formed, along with the continuously stirred solution flowing over the membrane surface. The membranes employed were of cylindrical geometry (ID = 8mm, OD = 14mm), with average pore size of 1.2 µm (process C) and the total surface area available to create emulsion was approximately 31 cm^2 (fig. 6.2). The feeding strategy was kept the same as in the conventional process (process A). The transmembrane pressure required to obtain the flux of 96 l/ (m^2. h) was 2.6 and 1.6 bars in process B and C respectively. The

operating pressure and flux rate influence the droplet size because of the coalescence at the surface [Joscelyne and Traegardh, 1999]. This effect was planned to be quantified and optimized later.

Fig. 6.2: Cylindrical ceramic membrane fitted in the bioreactor for emulsified feeding of oil.

6.3.4 Effect of emulsification on biomass and product

The results are graphically presented in figure 6.3. It can be seen that with a 0.2 µm membrane (process B) the biomass as well as the rhamnolipids production were negatively affected. The reason for this failure could be that although the resulting emulsion was stable, the oil droplets were too small for the cells to feed upon, rather they were blocking the cell surface [Angelova and Schmauder, 1999] and hence had negative effect on cell metabolism. Moreover, because of the very small pore size used, the emulsion was very fine and hence led to high viscosity (~ 9 cSt) which affected the normal functioning of cells in the medium negatively.

With a 1.2 µm membrane (process C), the biomass grew to the same level as in process A. No problem was faced with respect to viscosity, which was observed in the normal range of 2-3 cSt. After 60 hours, this fermentation was carried out at lower aeration (0.1 LPM as compared to 0.3 LPM in process A). The comparable biomass growth justifies the perception that homogeneously dispersed oil helps in enhancing the dissolved oxygen in the reaction mixture. However, the rhamnolipids production was limited to only half the total amount as achieved in process A.

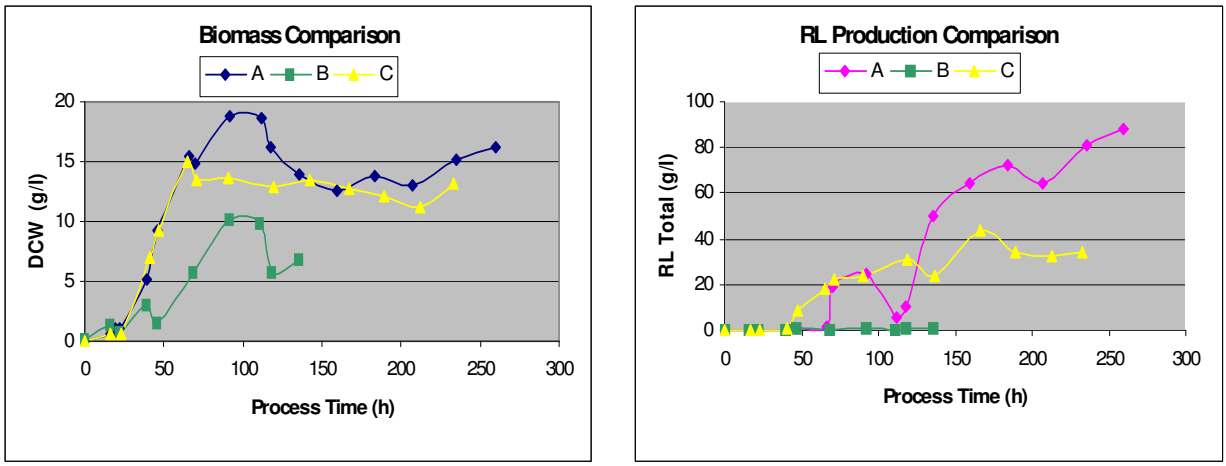

Fig. 6.3: Comparison of biomass growth and rhamnolipids (RL) production in process A, B and C.

The failure to convert the biomass growth into rhamnolipids production could only be justified with the available know-how about the biotenside production, which states that microbes are known to produce surfactants and thus render hydrophobic compounds more bioavailable [Karsa and Porter, 1995]. This means that surfactants are produced by the bacteria to increase their own reach to the substrate by enhancing its dispersion into the medium. Thus, when the substrate is already dispersed homogeneously, as during emulsified feeding, they do not need to produce the surfactants, or the need is reduced if not completely ruled out.

6.3.5 Stability of membrane system

It is very important that the integrated membrane modules should not have any negative effect on the bioprocess and should be stable during the long run as well as during autoclave (sterilization). The ceramic membrane modules showed remarkable stability against the continuous shear during the long process, as well as against high temperature and pressure during autoclave (fig. 6.4).

At the end of the process, the epoxy adhesive showed a problem when autoclaved in presence of surfactants: the adhesive lost binding with the membrane surface (fig. 6.5). This could be due to the seepage of some amount of oil into the epoxy material, which could have been avoided if the membrane was sealed at the sides prior to potting. Furthermore, some unknown reactions of the epoxy resin with surface active reagents can not be ruled out. The aforesaid problem was not encountered when the coated membrane was autoclaved only with oil. Separate tests confirmed this result.

Hence, the same adhesive can be used for the purpose, right from the beginning, throughout the process and the small membrane just needs to be potted again before the next run. For higher scales, the membrane modules are fitted within stainless steel casings and the adhesive is not required then. Thus, overall this system offers a better alternative to external systems used for emulsification in aqueous two-phase systems with respect to cost-effective, sterile, simple and continuous working.

Fig. 6.4: Integrated ceramic membrane at the end of the process.

Fig. 6.5: The epoxy adhesive lost binding when autoclaved with surfactant.

6.3.6 Analysis of emulsion

It is interesting to note that even in the conventional process, as more and more rhamnolipids are produced and secreted into the reaction mixture, the remaining oil would get more and more homogeneously dispersed and thus form a stable emulsion. Therefore, there should be a state of emulsion, depending on the concentration of oil and rhamnolipids in the reaction mixture, which is optimal for rhamnolipids production as well as at some stage; there should not be any production at all. This trend could be observed at the end of process A, where the biomass is maintained at the highest level (for this system) but without any further significant rhamnolipids production. Assuming that in a stable emulsion, the dispersed oil droplets in aqueous medium are like insoluble and non-reacting particles dispersed in a medium, the emulsion within the reaction mixture could be

quantified in terms of particle-size distribution corresponding to the droplet-size distribution.

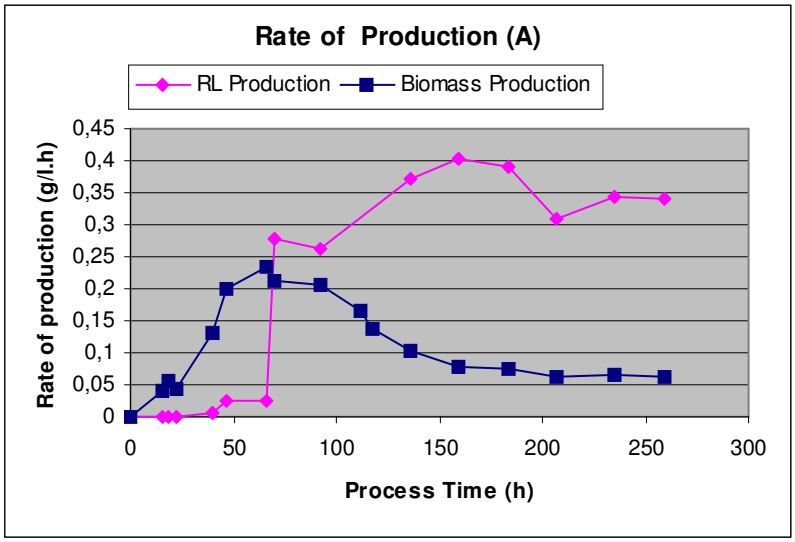

Fig. 6.6: Cumulative Rate of biomass and rhamnolipds (RL) production in Process A.

To quantify the emulsion at different process times, it was important to note which process points are most significant regarding to rhamnolipids production. For this purpose, the rate of production for biomass and rhamnolipids in the conventional process was graphically observed (fig. 6.6). For analysis, following process points were chosen (table 6.1):

- the point when the feeding was started (1) and when it was finished (2), as the rate of substrate addition in process A was very slow.
- the point when the rhamnolipids production shot up (3) (in less than five hours the production went from 1.6 to 19 g/l).
- the point until which the rate of production kept increasing (4) (until 159 hours after which the production continued but the rate of production decreased).
- and finally, at the end of the process (5).

Also, feeding with a 0.2 µm membrane (6) and a 1.2 µm membrane (7) was analyzed at the same rate of mixing (600 rpm) and at same TMP as in process B and C.

The experimental set-up was simulated for 500 ml cell-free volume with the same concentration of oil and rhamnolipids as in the actual bioprocess at that process-time

(table 6.1). The comparative distribution is graphically presented in figure 6.7 and the results for droplet-size distribution are summarized in table 6.1.

Table 6.1: Droplet size data for different process points and for emulsified feeding via membranes.

S. no.	Emulsification (Process time)	Conc. Oil (g/l)	Conc. RL (g/l)	Avg. Droplet Size (μm)	Distribution Range (μm)
1	Conv., 600 rpm (46 hrs)	80	1.2	65.65	0.37 – 146.8
2	” (65 hrs)	175	1.6	92.99	0.37 – 213.2
3	” (69.8 hrs)	150	19	74.9	0.37 – 121.8
4	” (159 hrs)	133	64	15.1	0.37 – 52.63
5	” (260 hrs)	106	88	2.37	0.37 – 5.61
6	0.2 μm membr., 600 rpm (45 hrs)	80+125	0.8	1.83	0.37 – 5.61
7	1.2 μm membr., 600 rpm (45 hrs)	80+125	4.2	3.55	0.37 – 8.14

6.3.7 Comparative analysis

The graphs (fig. 6.7) show that the distribution of oil droplets gets narrower as more and more rhamnolipids are produced. The average droplet size is the least at the end of the process (sample 5), when there is no further production. At the end of the process, the distribution is more or less similar to the distribution achieved via membrane (sample 7). Hence, it could be concluded that at this stage, surfactants production was not required. However, the biomass level was maintained which means that the substrate was still available. With the 0.2 μm membrane, the distribution range is similar as it is shown at the end of process A. Nevertheless, the average droplet size is less. Moreover, there are finer droplets which might have been responsible for the negative effect observed. With the 1.2 μm membrane the distribution as well as the average droplet-size is a bit larger; this would have lead to the rhamnolipids production in the beginning. Later, with more rhamnolipids, the same state of emulsion might have been reached as observed at the end of process A. More interestingly, the average droplet size as well as the distribution is more or less similar for sample 1 and 3. However, the difference in productivity is due to the total amount of oil present.

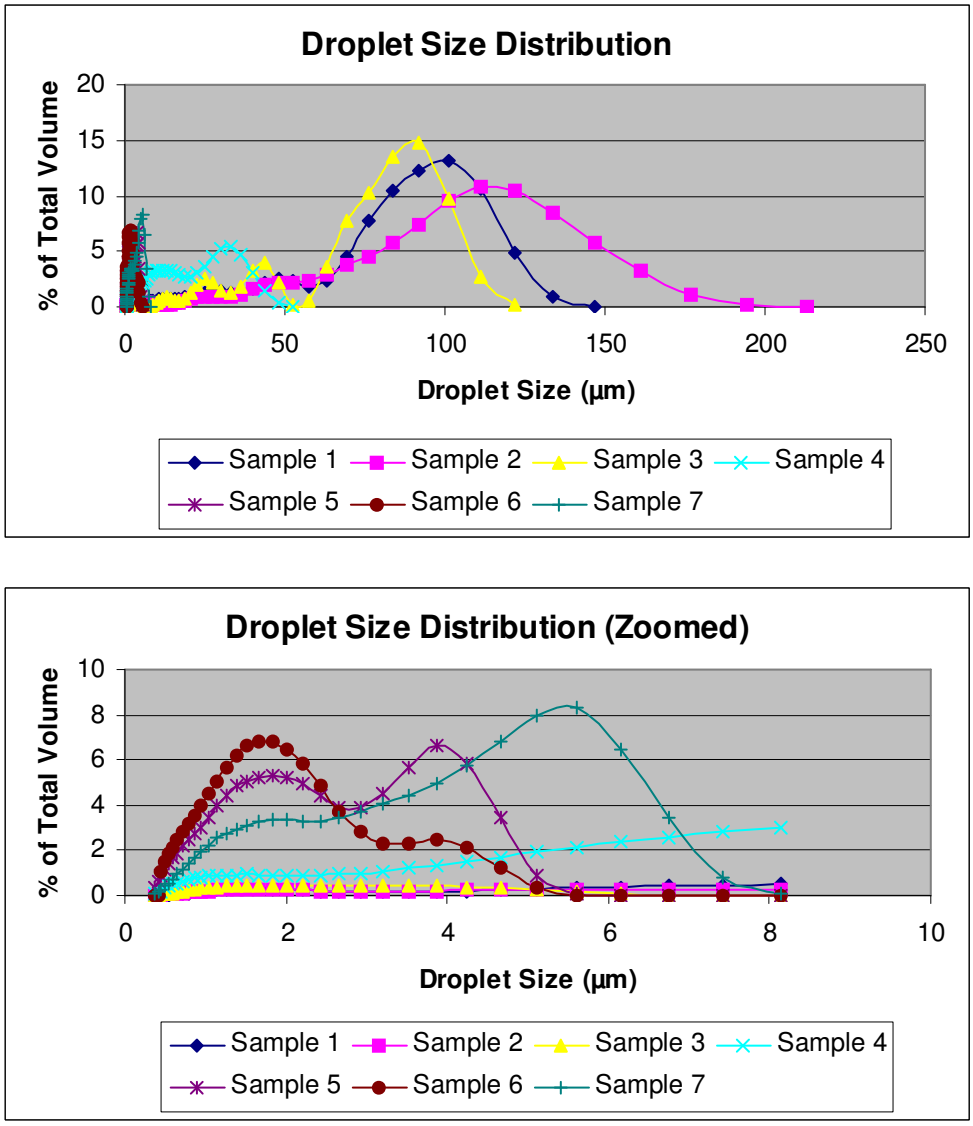

Fig. 6.7: Comparison of droplet-size distribution at different process times as well as with emulsified feeding via membranes (top); zoomed view of the graph up to 10 µm droplet size (below).

Although these simulated results can not depict the actual state of emulsion in the bioprocess nor variations due to coalescence during sample preparation could be ruled out, this analysis for droplet-size distribution gives a fair understanding of rhamnolipids production at different process points. Thus, we can conclude that the optimal droplet size for rhamnolipids production in this system lies somewhere between 70 and 90 µm. It should be noted that smaller droplets for the same amount of oil show that oil is more homogeneously dispersed and that more surface is exposed to the aqueous medium. Hence, if the droplet size can be limited to around

2-3 μm for a given amount of oil in the medium, the surfactant production could be very well minimized, if not completely ruled out.

6.4 Bioconversion of Waste Oils

6.4.1 Background and Objective

Polyhydroxyalkanoates (PHA) are the raw material for the production of biodegradable plastics. Some of the PHA have similar properties to polypropylene and could be a preferred choice owing to their biodegradability. Moreover, it is also their synthesis from renewable carbon sources, based on agriculture or even on industrial wastes which make PHA so fascinating [Braunegg et al., 1998]. Major limitation to the commercial production of PHA to replace petrochemical polymers is the higher overall costs of the bioprocess [Kleerebezem and Loosdrecht, 2007]. To date only one company (Monsanto, USA) has offered a range of PHA produced with the bacterium *Ralstonia eutropha* under the trademark BIOPOL®.

A process for production of PHA with *Pseudomonas aeruginosa* 42A2 (NCBIM 40045) using waste oils as carbon source has been developed at the Department of Microbiology and Parasitology, University of Barcelona, Spain [Guerrero et al., 1997]. In this process the oleic and lenoleic acids present in the waste oils are transformed by the bacteria into a mixture of PHA consisting of: (E)-10-hydroxy-8-octadecenoic acid, (E)-7,10-hydroxy-8-octadecenoic acid (E)-10-hydroperoxy-8E-octadecenoic acid [Cullere et al., 2001]. The composition of PHA produced by *Pseudomonas aeruginosa* 42A2 is substrate dependent and functional groups, for example double bonds, may be incorporated into the biopolymers. This feature allows the production of tailor-made biopolymers [Bassas et al., 2006].

The duration of the conventional batch process for PHA production is 48 hours, during which 20 g/l of oil (fed in the beginning after inoculation) is consumed and about 22 g/l biomass (dcw) is produced along with approximately 5 g/l of the product. High amount of foam production is a major problem faced with this process (fig. 6.8), which not only affects the sterile working but also the accurate analysis of the process, due to significant loss of culture volume that leaves the reactor along with exiting foam. Employment of antifoam disk has not been helpful even when using

only 50% of the working volume. At the end of the process, a high amount of biomass is found sticking at the inner side of the top metal cover of the bioreactor (fig. 6.8). Thus, a lot of biomass is lost from the reaction solution which would otherwise contribute to the bioconversion and hence to the overall efficiency and cost-effectiveness of the bioprocess.

Fig. 6.8: The foaming problem during the batch process for PHA production with *P. aeruginosa* 42A2: the bioreactor is full with foam even when employing only 50% working volume (left), most of the cells reach the top cover of the reactor with the foam and stick there forming biofilms (center), the overhead column connected to the exit gas port for collection and breaking of foam is completely full with foam and a lot of foam flows out and could not be recycled back to the bioreactor (right).

Based on our earlier experience with *aeruginosa* culture (as discussed in section 6.3.4), emulsified feeding of the oily substrate via reactor integrated ceramic membranes was investigated. The objective was to achieve a feeding strategy that should minimize the foam production while enhancing the uptake of substrate and hence the bioconversion by the cells. For this purpose it was important to define the parameters with which the desired emulsion could be achieved. The methodology for the same is discussed in the next section.

6.4.2 Optimization of process factors

As discussed in section 6.3.7, if the average size of oil droplets in the aqueous medium could be limited to 2-3 μm, surfactant production and hence foaming could be controlled. It was also observed that pore size of the employed membrane had influence on emulsion produced at same feeding rates via the membranes. Thus it

was decided to investigate for an optimal pore size and also the optimum TMP to produce the desired emulsion with a narrow droplet size distribution.

Tests were made with cylindrical ceramic membrane of average pore size 800 nm (ItN Nanovation AG). The emulsification set-up as discussed earlier in this chapter was established for feeding 20 g/l of oil via the membrane into the aqueous medium. The solution contained 0.1 g/l of rhamnolipids and the agitation was set at 500 rpm. The ceramic membrane employed had a surface area of 17.6 cm^2. Different feed rates (which were possible with standard setting of the employed centrifugal pump) were investigated (the corresponding TMP data is summarized in table 6.2).

Table 6.2: Droplet size distribution for feeding via 800 nm membrane at 500 rpm.

Sample no.	TMP (bar)	Flux ($l/m^2.h$)	Avg. droplet size (µm)	Distribution range(µm)
1	only 20 g/l oil		no reproducible analysis	
2	only mixing		80.2	0.37 – 161.2
3	0.80	29.9	26.4	0.37 – 76.4
4	1.50	57.5	28.9	0.37 – 69.6
5	0.45	14.6	30.3	0.37 – 92.1

*except the first all other test solutions contained 0.1 g/l emulsifier.

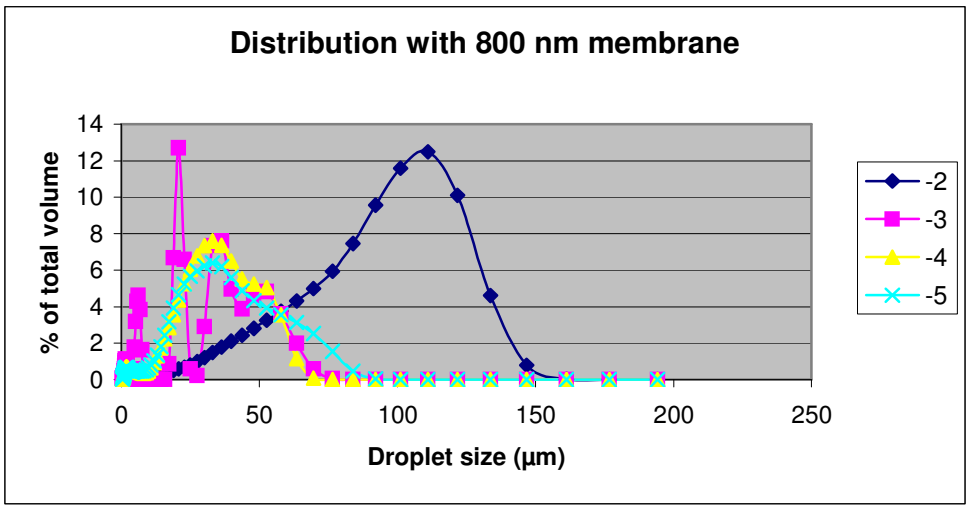

Fig. 6.9: Droplet-size distribution for emulsions achieved with feeding via 800 nm membrane at different feeding rates. Distribution 2 was achieved by mixing-only the test solution at 500 rpm. Technical data regarding emulsification is summarized above in table 6.2.

Droplet size distribution for a mixture of oil and aqueous medium without any surfactant should have been considered as reference. However when investigated, it was not possible to analyze such a mixture with the method used for this study due to formation of a distinct separate layer of oil as soon as the sample was collected out of the agitated solution. The distribution varied too much for different samples from the same set and hence the results were not reproducible, which is important to accept the analysis. Hence it was concluded that a minimum about of emulsifier (surfactant) is required in the solution to provide a stable emulsion for analysis.

As can be observed in figure 6.9, the distribution achieved with addition of oil through the submerged membrane at different TMPs was narrower as compared to what was achieved with mixing-only at 500 rpm without using the membranes. Though the difference in employed feed rates and the corresponding TMPs was significant, no significant difference in average droplet size or the distribution range achieved with emulsified feeding could be observed (table 6.2). Perhaps the concentration of the emulsifier was not sufficient enough to hold the emulsion as it was produced at different settings of feed rate and TMP. Hence, it was decided to repeat the investigation for the same amount of oil and at the same agitation but with higher surfactant concentration. Cylindrical-ceramic membrane of average pore size 400 nm was also included in this series of tests. The employed membranes had a surface area of 22 cm^2. Different feeding rates were investigated and are summarized along with the corresponding TMP values in table 6.3.

As illustrated in table 6.3 and figure 6.10, the most optimal droplet distribution, as desired for our purpose, was achieved with 400 nm (avg. pore size) membrane for a flux of 20.8 $l/m^2.h$ at a TMP of 0.95 bars. Also, the effect of higher TMP as well as lower pore size of the membrane could be seen in the results. The average droplet size decreases in the beginning but eventually rises with increasing flux and TMP, thus indicating higher coalescence under those conditions. Moreover, for nearly the same TMP values the flux achieved with 800 nm (avg. pore size) membrane was slightly higher than what was observed in the previous case with less emulsifier (table 6.2). Perhaps higher concentration of emulsifier in the solution enhances the detachment of oil droplets from the membrane surface and hence the flux.

Table 6.3: Droplet size distribution for feeding via ceramic membranes at 500 rpm and 1g/l emulsifier.

Emulsion no.	Emulsification mode			Avg. droplet size (µm)	Distribution range (µm)
	Pore size (nm)	Flux ($l/m^2.h$)	TMP (bar)		
E1	Mixing-only without membranes			57.5	0.37 – 234.1
E2	800	33.4	0.85	5.60	0.37 – 8.14
E3	800	53.7	1.30	4.40	0.37 – 8.14
E4	800	84.6	1.80	6.55	0.37 – 7.42
E5	800	15.1	0.45	7.71	0.37 – 8.90
E6	400	20.8	0.95	2.45	0.37 – 5.11
E7	400	43.2	1.40	1.73	0.37 – 4.24
E8	400	60.6	1.75	1.45	0.37 – 2.66
E9	400	13.5	0.70	2.80	0.37 – 8.14

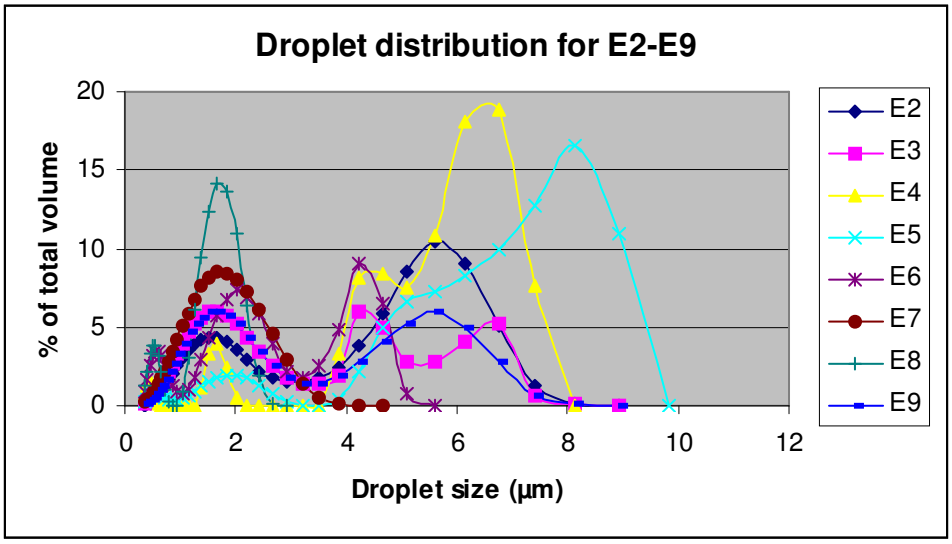

Fig. 6.10: Droplet-size distribution for emulsions achieved with feeding via different ceramic membranes at different feeding rates. Technical data regarding emulsification is summarized above in table 6.3. Distribution for E1 achieved by only mixing of the test solution at 500 rpm is not included.

6.4.3 Emulsified feeding for the production of PHA

In the conventional batch process for the production of PHA, 20 g/l of oily substrate is fed in the beginning after inoculation of the culture. However as the complete consumption of oil takes place over 48 hours of process time, it was decided to feed the oil through the submerged membrane in pulses of 10 g/l. This was done to ensure better emulsification effect due to reduction in coalescence of oil droplets as

less amount of free oil would be present in the culture solution. With the available pump, it was not possible to fine tune the flow rate and hence to achieve a TMP of 0.95 bars and a flux of 20.8 l/m^2.h, which was found optimum (table 6.3) to avoid surfactant production. The feeding was performed with a flow rate of 1 ml/min, which corresponds to a flux of 22 l/m^2.h for the employed membrane at TMP of approximately 1 bar. Amount of oil present in the solution was checked with regular sampling, and the next pulse of substrate was given only when no distinct oil layer was observed after centrifuge. This was done to avoid the coalescence of oil in the solution, however any limitation of the carbon source was also checked.

6.4.4 Effect of emulsified feeding on the bioprocess

Fig. 6.11: Emulsification via submerged membrane, fine oil droplets could be seen here coming out at the membrane surface.

Fig. 6.12: Effect of emulsified feeding; the foam production was significantly reduced and hence the loss of culture volume was completely controlled.

As can be seen in figure 6.11, emulsified feeding of oil was comfortably achieved via the integrated membrane. Total 5 feed pulses of 10 g/l of oil were given at zero hour (after inoculation), 3 hours, 15 hours, 23 hours and 43 hours of process time. The foam production was significantly reduced (fig. 6.12) and hence the loss of the culture volume was completely checked. The biomass and PHA production during the 50

hours long process are shown in figure 6.13 and are compared with the results from the conventional batch process.

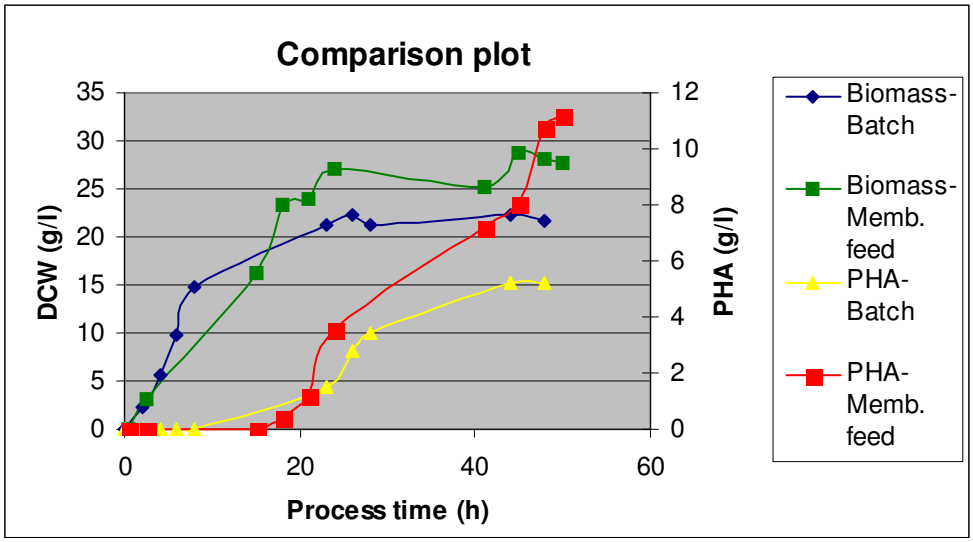

Fig. 6.13: Biomass and PHA production in batch and membrane process: the biomass production was a little higher in membrane integrated process but the PHA production was more than double as compared to the batch process.

As can be seen in figure 6.13, the biomass growth was slightly slower in case of membrane integrated process, but the final concentration was slightly better as compared to the batch process. Moreover, the production of PHA was more than double to what was achieved with the batch process. It is to be noted that PHA production was mainly achieved after 22 hours of process time after which the biomass concentration was more or less constant, while the PHA production rate jumped significantly after 4^{th} and 5^{th} pulse of substrate. However, the production did not correlate with the amount of substrate fed during the process. A thick layer of EPS (which have not been characterized in this study) was observed when the samples from the bioreactor were centrifuged. Thus, a lot of substrate was not converted to the desired product. Nevertheless, it could be observed that with emulsified feeding a relative excess of the carbon source (oil) in the culture could be obtained, which is important for PHA production [Bassas et al., 2006]. In the same process time, higher amount of substrate could be consumed by the cells resulting in a higher production and productivity, which could further be improved by engineering the microorganism to produce less EPS and more of the desired product.

6.5 Conclusion and outlook

A method for substrate feeding in aqueous two-phase fermentation process has been demonstrated by emulsification within the bioreactor volume using submerged ceramic membranes. The technique is attractive given the low energy consumption, the better control of droplet size distribution and especially the mildness of the process. Emulsified feeding of oil via reactor-integrated ceramic membranes has been investigated both for production of rhamnolipids, as well as to minimize the surfactant production, and hence the foam in *P. aeruginosa* fermentations developed for different purposes.

It is very important for the significance of any engineering method to have the suitability to scale-up, and the potential to reproduce similar or better results as achieved at bench scale. Although emulsified feeding via submerged membranes has not been advantageous for rhamnolipids production, it could still be a potential application when considering higher scale operation for the same purpose. It should be noted that at bench scale (2 l volume) the rate of mixing was kept very high (600 – 900 rpm) due to the employment of antifoam disc. At a higher scale, to attain the same level of agitation the energy input per unit volume (P/V) would need to be increased. Furthermore, if a suitable method as bubble-free aeration and continuous removal of CO_2 from the culture (for foam reduction) could be developed [Mohrdieck, 2007] high agitation would not be required when working with higher volumes.

However, with lower rpm the mixing time will increase which would otherwise affect the production. In that case it would be important to achieve an optimal droplet distribution at lower agitation rates. The submerged membrane system can provide some '*assistance*' to the mixing to achieve the droplet size distribution that is optimal for rhamnolipids production, while keeping the energy input low. As a proof of concept, investigations were made using a ceramic support of average pore size 5 µm (inopor GmbH, Germany) for feeding 100 g/l oil in aqueous solution. The aqueous medium had 1 g/l of rhamnolipids and the mixing rate was set at 100 rpm. Emulsion achieved with mixing-only of the solution without using membranes is taken as reference for comparison to what is achieved via feeding through the membrane. The data is summarized in table 6.4 and is graphically presented in figure 6.14.

Table 6.4: Droplet size distribution for feeding via 5µm membrane at 100 rpm.

Sample no.	TMP (bar)	Flux ($l/m^2.h$)	Avg. droplet size (µm)	Distribution range(µm)
1	only mixing		184.1	0.37 – 194.2
2	0,9	230	92.6	0.37 – 133.7
3	1.25	323	76.4	0.37 – 146.8

* all test solutions contained 1g/l emulsifier.

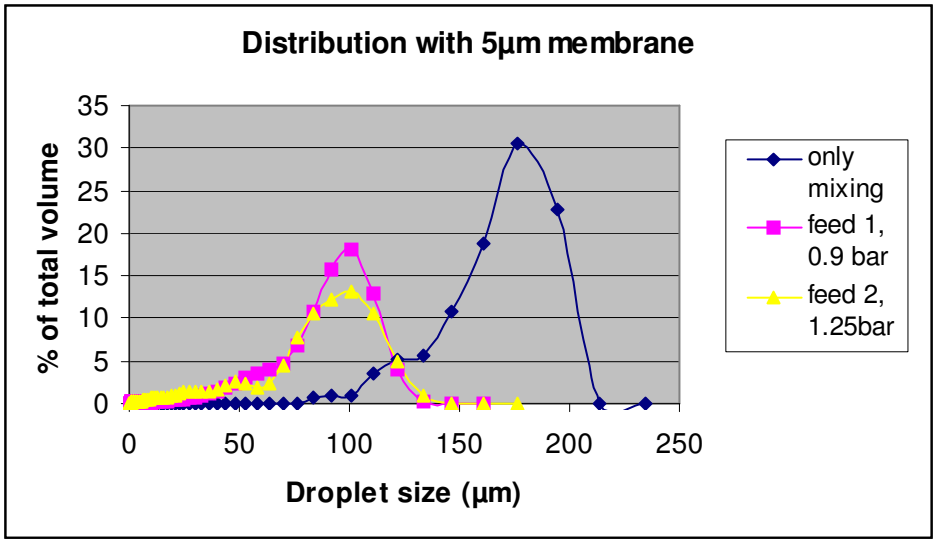

Fig. 6.14: Droplet-size distribution achieved with feeding via 5µm membrane at different feeding modes, distribution achieved by mixing-only of the solution at 100 rpm is shown for comparison purpose.

As could be observed in figure 6.14, by using a ceramic support submerged in the reaction solution a suitable emulsion for rhamnolipids production could be achieved even at low agitation. Due to larger pore size of support matrix, high feed rates could be achieved at low transmembrane pressures and hence energy input could be kept low. Thus, an optimal mixing could be achieved in a cost-effective way as compared to external cross-flow methods [Graaf et al., 2005] reported for the same purpose.

Moreover, the emulsified feeding through submerged ceramic membrane system had been advantageous in terms of better substrate uptake and control of foam in aqueous-two-phase system. In the process developed for production of polyhydroxyalkanoates (PHA) with *P. aeruginosa*, substrate consumption and hence the bioconversion was improved while reducing the foam production. Furthermore, strain improvement and optimization of the feeding strategy would be investigated.

7 Hydrophobization: polymer coating on ceramic surface

7.1 Introduction

Ceramic membranes offer unique advantages over their polymeric counterparts owing to their high mechanical and chemical strength [Hsieh et al., 1991]. However, in certain cases such as separation of products from organic-aqueous (oil-water) mixtures the applications are limited due to the hydrophilic character of the membrane. Irreversible fouling due to adsorption of a wide range of solute species present in oily streams can severely reduce membrane efficiency and lifetime [Faibish and Cohen, 2001]. On the other hand, polymeric membranes lack the mechanical strength, especially when the submerged application is desired. A suitable combination could be a membrane that offers selectivity of the hydrophobic membranes and at the same time mechanical and thermal stability of the ceramic membranes. Studies have been reported where modification of the ceramic membrane system is carried out either by modification of ceramic particles [Eljaouhari et al., 2006], or by treatment of ceramic surfaces with hydrophobic solutions [Geerken et al., 2007]. Due to the technical difficulties associated with the preparation of ceramic membranes and our limitation in doing so, post membrane surface modification methods are investigated in this study. The aim was to obtain a thin layer of hydrophobic entity over a porous ceramic support, thus developing a hydrophobic membrane with high mechanical and thermal strength. The coating method employed for this study has been described in chapter 3. The membranes made available for this study were ceramic monochannel supports (ID=8mm, OD=14mm) with average pore size between 600 to 800 nm and were generously supplied by ItN Nanovation AG, Germany.

7.2 Hydrophobization with PTFE

Polytetrafluoroethylene (PTFE) is commonly known as Teflon (DuPont brand name) and is known for its hydrophobicity and high melting temperature. ItN Nanovation AG have been using PTFE suspensions for coating the ceramic membrane for sealing purposes. PFA 6900 RG dispersion (Dyneon GmbH, Germany) based on PTFE was

suggested for the coating purposes. Furthermore, dense coating of PTFE on ceramic membranes has been successfully investigated for gas exchange in the fermentation medium [Mohrdieck, 2007]. However, this coating method needs to be tested again for the specific purposes and applications. In this study, the objective was to employ a very thin layer of polymer on the hydrophilic surfaces of the ceramic membrane and hence to obtain a microporous hydrophobic membrane with high mechanical and thermal strength. It was important that not only the outer/inner surface but also the complete inner matrix should be coated with the thin polymeric layer.

As it has been already shown that ceramic membranes have a wide range of pore distribution, it was important to confirm the size distribution of the PTFE particles in the suspension. In the PFA 6900 RG dispersion (50% w/v), PTFE particles were expected to be on average 230 nm in size (manufacturer's details). However, when analyzed for a 0.1% PFA suspension the particle size distribution ranged from 0.37 to 1.0 μm. It is to be noted that the minimum value that could be analyzed by the particle counter device (LS100 particle size counter, COULTER, USA) was 0.37 μm. Hence, there was a significant agglomeration among the single particles to form bigger particles. Thus, the coating method that was successful when applied only on the surface to put a dense layer would have failed if investigated to thin-coat the whole membrane. The failure was expected due to the blockage of the pores at the surface with bigger agglomerates and blockage of the porous matrix with single particles and/or smaller agglomerates.

Thus, the higher concentrations of PTFE in dispersion would have reduced the filtration area at the surface and consequently the flux, while the lower concentrations would have reduced the porous area inside the membrane matrix and hence the flux. Hence, further investigations regarding hydrophobization of ceramic membranes with PTFE were ruled out.

7.3 Hydrophobization with alternate solutions

The coating with PTFE was not promising due to agglomeration of particles in suspension. Apart from PTFE, polymeric membranes made of polypropylene glycol (PPG) are commonly used. For these tests, the concentration of PPG was set at

0.001% (w/v) as has been optimized in a separate study at upt GmbH [Mohrdieck, 2007]. Furthermore, as surfactants are known to have a hydrophobic (the tail) and a hydrophilic (the head) moiety in their structure [Holmberg et al., 2003], one set of tests was also performed where a solution of sodium-dodecyl benzylsulfonate (BAS) was used for coating the ceramic membranes. The concentration of BAS was set at 30 ppm, as higher concentrations showed unclear emulsions with water. The coating procedure and the membranes were same as discussed before and the hydrophobization was estimated with pure water flux and inert gas flux.

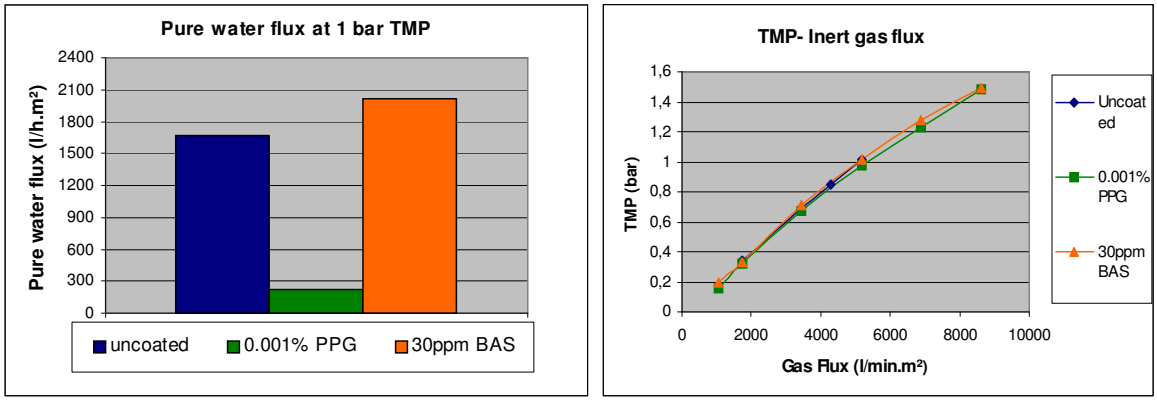

Fig. 7.1: The water flux of non-coated and coated membranes (left), the type and the concentration of the coating solution is shown. The inert gas (N_2) flux of the non-coated and coated membranes(right), no difference in TMP for similar inert gas flux but reduction in pure water flux showed that hydrophobization has been achieved.

As can be seen in figure 7.1, pure water flux for the membrane coated with PPG was much lower than that of the reference (non-coated) membrane. However, the inert gas flux showed a similar pattern as observed for the reference membrane. Thus, confirming hydrophobization and lack of significant blockage of the membrane matrix. It is to be noted that binding of PPG molecule with ceramic surface is based on O—H bond formation with Al_2O_3. The inert gas flux with the BAS coated membrane also showed a similar pattern; however, the pure water flux was slightly higher than that of the reference membrane. The only justification for such an effect could be the formation of a double layer of surfactant, where the hydrophobic tail of the surfactant compound binds with the hydrophobic tail of another molecule and hence exposing the hydrophilic ends, one of which binds with the membrane and the other is free [Holmberg et al., 2003]. Nevertheless, filtration of alginate solution was carried out with both the coated membranes. Alginate has been used as a model extracellular

polymer for membrane fouling studies [Ye et al., 2005] and investigations have been reported for filtration of alginate with ceramic membrane [Cheze-lange et al., 2002]. Fouling of ceramic membrane with alginate has been attributed to the hydrophilic character of both the membrane and the filtrate compound, and the same has been observed in the experiments earlier conducted by us (chapter 5).

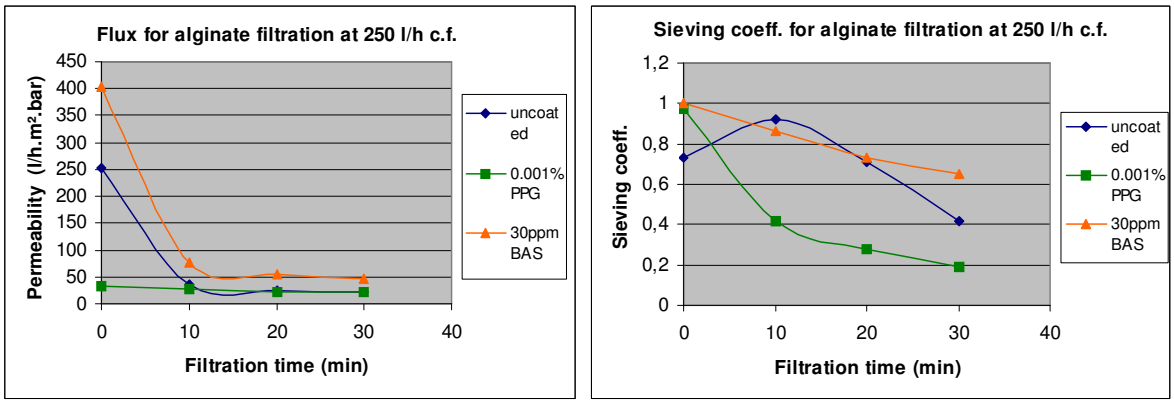

Fig. 7.2: Flux during alginate filtration with 250 l/h of the cross flow feed circulation (left) and sieving coefficient of alginate with filtration time in the same experiment (right). The reduction showed that the membranes, coated or non-coated, were gradually fouled with alginate during the course of filtration.

As can be observed in figure 7.2, the coated membranes depicted a similar flux pattern for the alginate solution fed at 250 l/h cross flow rate. The TMP at this flow rate was observed between 0.9 and 1.0 bars during the investigations. The flux was better for the BAS coated membranes as compared to the PPG coated membrane in the beginning, but eventually a similar value was obtained for both the membranes. On the other hand, the sieving coefficient of alginate showed an interesting pattern. The decline in permeation of alginate was observed, however gradual as compared to the observation with non-coated membrane. Next, filtration was carried out at 600 l/h cross flow feed rate and the results are depicted in figure 7.3. The TMP at this flow rate was observed between 2.6 and 2.8 bars during the investigations. As can be seen, the decline in flux followed a similar pattern to that observed with non-coated membrane, but the sieving coefficient of alginate constantly remained at a high level. Hydrophobization of ceramic membranes with PPG and BAS to separate organic phase from stable emulsions is a matter of detailed discussion in a separate doctoral work [Mohrdieck, 2007].

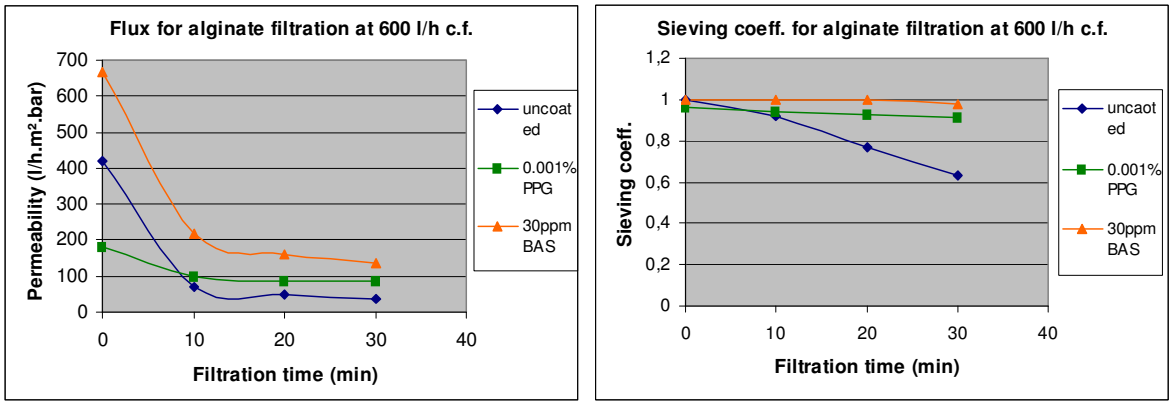

Fig. 7.3: Flux during alginate filtration with 600 l/h of the cross flow feed circulation (left) and sieving coefficient of alginate with filtration time in the same experiment (right). The reduction in flux while consistency in sieving coefficient showed that the coated membranes were being hydrophobisized during the filtration.

7.4 Conclusion and outlook

Methods for hydrophobization of the hydrophilic ceramic membranes have been investigated in order to enhance their selectivity and hence the range of their applications. The coating of ceramic membranes with Polytetrafluoroethylene (PTFE) were ruled out due to agglomeration of particles in the suspension and hence the expected blockage of the membrane surface and the inner matrix. However, when solutions of polypropylene glycol (PPG) and sodium-dodecyl benzylsulfonate (BAS) were used for coating the results were quite promising. Investigations with PPG and BAS would be continued with the aim to establish a stable, easy and cost-effective method for hydrophobization of the ceramic membranes. Successful hydrophobization while maintaining high flux characteristics would add a whole new range of applications for ceramic membranes, especially in the submerged form where polymer based membranes are not suitable due to their limited mechanical strengths.

8. Summary and Outlook

In this work, different applications for ceramic membranes integrated with the bioreactor in submerged form have been investigated. Membranes integrated within the bioreactor (i.e. in submerged form) are advantageous over external membrane systems in terms of space and energy requirement and most importantly by providing sterile operation. Unlike in the case with external systems, extra culture volumes are not demanded and lack of oxygen in the circulation loop is not faced with submerged systems. These features are very significant when highly aerobic and sophisticated cultures are involved. In this work, the processes to which submerged ceramic membrane systems have been employed are fermentation processes developed for the production of various compounds having vital applications in pharmaceutical, detergent, biopolymers and food industry. The results from this work can be summarized as:

- Ceramic membrane systems can be integrated within the fermenter for complete biomass retention (*P. pastoris*) inside the bioreactor and product (lipase) recovery in the permeate. Thus, the first step in down stream processing could be integrated with the production process and the operation could be carried out in a (semi) continuous, sterile and cost-effective manner. Furthermore, the submerged membrane system has been successfully investigated for repeated batch process with *S. pombe*. With the membrane process the overall biomass production and hence the biotransformation of specific substrates to human metabolites could be improved up to 8 times as compared to the conventional batch process.
- Filtration of bacterial culture (*P. aeruginosa*, *P. fluorescens*) and recovery of products (rhamnolipids and PHAs) with submerged ceramic membrane systems have not been successful due to severe fouling of submerged membranes with high amount of EPS produced by these cultures. Different methods have been investigated to control the fouling, but failed due to presence of hydrophilic as well as hydrophobic foulants in the production culture. Nevertheless, biomass retention and product recovery has been successfully shown with *C. glutamicum* culture used for production of lysine, an essential amino acid. Hence, the

submerged system could be employed for high cell density bacterial fermentation with cultures that do not produce biofilms.

- Emulsified feeding of oily substrate into the aqueous medium via submerged membranes has been investigated for aqueous-two-phase systems developed for the production of rhamnolipids and PHAs. Interestingly, the emulsified feeding had opposite effects on the bioprocesses carried out with different species of *P. aeruginosa.* The rhamnolipid production was effected negatively when the substrate was fed through the membrane (with average pore size in range of 0.2-1.2 µm), however the PHA production was improved and foaming could be controlled in the membrane-integrated process. The emulsification method has been optimized in terms of pore size of the employed membrane and the TMP to achieve optimal droplet distribution in the aqueous medium.
- To enhance the range of applications, especially in submerged form, hydrophobization of the ceramic membranes has been investigated with different hydrophobic compounds. The aim was to develop a fine hydrophobic layer over a ceramic support, so that the hybrid membrane could be employed for selective recovery in the processes where normal hydrophobic membranes are not feasible due to their limited mechanical and thermal strength. Filtration of alginate has been carried out as a model study. To thus end, coating with PTFE was not feasible but coatings with PPG and BAS have been shown successful.

Hence, ceramic membranes could be integrated within the bioreactor for: biomass retention, product recovery and substrate feeding. Furthermore, it is also suggested that submerged ceramic systems can be used for aeration as well, however no specific investigation were carried out in this work pertaining to aeration purpose. One of the optimal strategies could be to use two parallel-submerged systems alternatively for product removal and aeration/substrate feeding. While the permeate is extracted through one set, the other is supplied with substrate/compressed air for back washing.

Although, all the investigations reported in this work have been carried out with bench scale bioreactors, the developed modules and the integration of membrane system is very well suitable for scale-up. Economical factors play a significant role

when large-scale operations are considered. These include longevity of the process, safety and cost of working with high pressure and mechanical load, cleaning and shelf life. Ceramic membranes have advantages over their polymeric counterparts in terms of mechanical and thermal strength as well as longer service life and easy cleaning procedures. Moreover, the production process as well as disposal after use is also environmentally friendly as compared to polymeric membranes.

Replacing external membranes with immersed ones, which began in the early 1990s, reduced costs (capital and operating) and increased the range of applications for which MBRs could be cost-competitive. Moreover, membrane costs have declined by an order of magnitude in the last decade, dramatically reducing MBRs costs. Several advances contributed to the reduced price, including development of better materials, more cost-effective configuration of membrane facilities, lower production costs because of greater economies of scale, more efficient production, and market place competition [Daigger et al., 2005].

Another approach to assessing the readiness of a technology for widespread application is the technology adoption cycle, which is a well-characterized process that can be described by an ‘S curve’. Adoption of a new technology is slow initially but picks up momentum as the new concept becomes more acceptable to a wider range of users. [Daigger et al., 2005]. Thus, good future prospects with wide spread applications and promising market could be predicted for submerged ceramic membrane systems. However, a lot of research work and investigations especially at pilot scale should be done in order to provide an acceptable solution for industrial bioprocesses. Moreover, investigations should be carried out to modify ceramic membranes with respect to biocatalyst immobilization and selective removal of components from a mixture. Owing to their unique ability to be integrated within the bioreactor, ceramic membranes hold a huge potential in the field of bioprocess engineering and biotechnology.

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