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Review

The Membrane Gradostat Reactor: Secondary metabolite production, bioremediation and commercial potential

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This manuscript focuses on the aspect of a membrane gradostat as an entirely different concept compared to submerged hollow fibre modules. The use of membrane bioreactor (MBR) technology is rapidly advancing in the wastewater treatment industries. However, this is not the case in the biopharmaceutical manufacturing industries. The MGR has shown great potential and versatility in terms of industrial applications. It can be used in both wastewater treatment and biopharmaceutical manufacturing using different modes of operation to meet any predetermined process requirements. The MGR concept uses capillary membranes, which contain microvoids in the substructure to immobilise microbial cells or enzymes, depending on the bioreactor's application. Operational requirements of the MGR and its commercial potential are discussed from a bioprocess engineering perspective.

Key words: Membrane bioreactor, gradostat reactor, secondary metabolite production, biofilm, wastewater treatment.

INTRODUCTION

Membrane bioreactors provide an environment for increased biomass density and improved productivity. The biomass immobilised in these systems is retained through a membrane barrier or support. Many membrane configurations have been tested, and the hollow fibre configuration is an interesting one (Charcosset, 2006). The production of secondary metabolites from immobilised biomass in biotechnology and pharmaceuticals, using membrane bioreactor systems, has not been widely deployed for production of low volume, high value bioproducts (e.g. the antibiotic, Actinorhodin). Novel developments and refinements in membrane technology continue to be active themes of research. The broad range of applications suggests that membrane technologies are now well accepted and cost effective (Wiesner and Chellam, 1999). In South Africa, membrane technology development tends to focus on water-related applications, although other membrane process applications are commercialised or are on the verge of being commercialised (Offringa, 2002). The use of membrane technology for biotechnological applications is therefore under exploited. By using this technology, alternative approaches to produce high value secondary metabolites continuously, can be a reality. Experiments indicated that most microbial biofilms are shear sensitive and would easily detach and slough off from membranes with relative smooth exterior surfaces (Jacobs and Sanderson, 1997). Researchers have also established that, some microbial cultures perform poorly in shear environments

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Abbreviations: MBRs, Membrane bioreactors; MGR, membrane gradostat reactor, ECS, Extra capillary space; SCMGR, Single capillary membrane gradostat reactor, and MCMBR, Multicapillary membrane gradostat reactor.

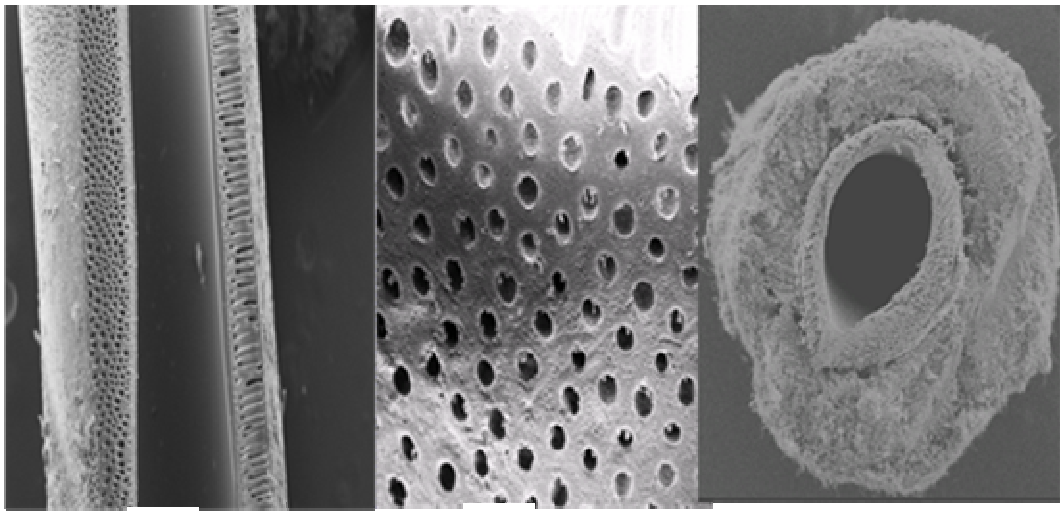

Figure 1. (A) SEM of a longitudinal section of the polysulphone membrane. (B) The external surface of the polysulphone membrane showing cavities where spores can be immobilised. (C) *P. chrysosporium* biofilm immobilised on the external surface of the polysulphone membrane.

where a shaker or aeration by bubbling is used. This clearly will make immobilisation of biofilms on external surfaces of membranes complicated. Until recently, extracellular enzymes from *Phanerochaete chrysosporium*, Lignin Peroxidase (LiP) and Manganese Peroxidase (MnP), were produced in stationary or shaken flasks and the supernatant was concentrated by centrifugation to recover the enzymes. Methods to produce these secondary metabolites continuously were developed, by immobilising biofilms of *P. chrysosporium* in membrane bioreactors (Venkatadri and Irvine, 1993; Leukes, 1999; Leukes et al., 1999; Solomon, 2001; Garcin, 2002; Ntwampe, 2005; Sheldon and Small, 2005). Generally microbial biomass was grown in the extra capillary space with nutrient medium flowing through the fibres, in these systems. Recently another geometry, where hollow fibres are inserted within another to grow the cells in the annulus between the two fibres was proposed (Yang et al., 2006). However, the concept of a membrane gradostat reactor is not understood. It is often confused with submerged hollow fibre membrane modules, which are commonly used in wastewater treatment applications. In this manuscript, the MGR is explained in detail. Other studies related to the use of the MGR are also reviewed.

The membrane gradostat reactor (MGR) concept

The original bidirectional compound gradostat reactor was described by Lovitt and Wimpenny (1981 a,b) and it has been simplified by making a more compact apparatus in which substrate gradients are established by diffusion between adjacent culture chambers (Lovitt and Wimpenny, 1981a,b). The devised gradostat had neighbouring vessels connected together by pumps in one direction and by overflowing weirs in the other. In the system, opposing chemical gradients were readily established. Wimpenny et al. (1992) designed a directly coupled gradostat consisting of different compartments. Unlike the conventional gradostat that uses pumps and weirs to transfer material between neighbouring vessels, the directly coupled gradostat relied on open transfer ports. However, the use of this type of gradostat proved to be costly, yielding low quality products and did not mimic natural living conditions of most microbial biofilms. A novel membrane gradostat reactor (MGR) was development by Leukes et al. (1999) and other researchers to immobilize *P. chrysosporium* biofilms to produce LiP and MnP using a polysulphone membrane (Jacobs and Leukes, 1996; Jacobs and Sanderson, 1997; Leukes et al., 1999; Solomon, 2001). Sheldon and Small (2005) showed that capillary ceramic membranes, with structural similarities to the polysulphone membrane were also suitable to immobilize *P. chrysosporium* biofilms in MGR systems. The term MGR is used to describe a biofilm reactor, which uses a synthetic capillary ultrafiltration membrane as a support matrix for the biofilm. The internally skinned and externally unskinned polysulphone membranes (Figure 1A and 1B), which were used, provided a unique substructure matrix within which a fungus of a filamentous nature could be immobilised on its external surface as shown in Figure 1C. Immobilising other microbial cell types, will be easier because of the microvoids in the polysulphone membranes. The substructure contains closely-packed narrow-bore microvoids (Figure 1A and 1B) that extended all the way from just below the internal skin layer to the membrane's external

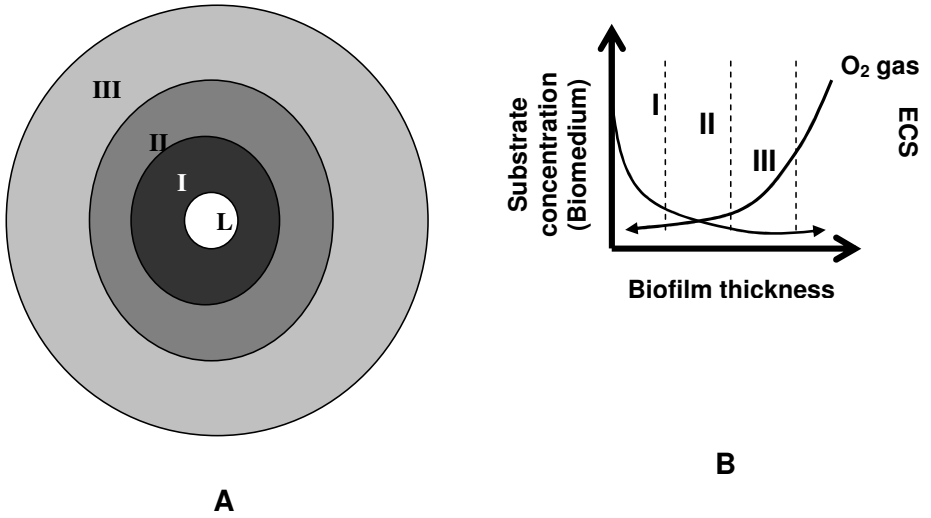

Figure 2. (A) Schematic representation of the membrane gradostat concept; I is the primary growth phase; II is the stationary growth phase; III is the decline phase; L- Lumen of the capillary membrane from which the nutrients are supplied. (B) Illustration of substrate concentration distribution and radial flow at different biofilm growth phases and thickness. Nutrient medium convective flow is opposite to the direction of oxygen diffusion, supplied in gaseous form on the shell side (ECS) of the MGR.

unskinned region. As a theoretical concept, the term membrane gradostat applies since the dissolved oxygen and liquid nutrient gradients flow is uni-directional and bi-directional with contact occuring between the primary (I), stationary (II) and decline (III) growth phases of the biomass (Figure 2). The nutrients are supplied at a rate, which is sufficiently low for a nutrient gradient to be established across the biofilm. The nutrient gradient is maintained such that it is sufficiently high enough to support primary growth of the microorganism and sufficiently low to induce secondary metabolite production in the biofilm further away from the substratum. The essence of this system is schematically illustrated in Figure 2A and 2B. When nutrients are supplied to the biofilm, which is immobilised on the external wall of a capillary membrane, bi-directional radial nutrient gradients are established across the biofilm. The biomass closest to the membrane has first access to the nutrients, while the biomass away from the membrane surface is starved therefore undergoing a ligninolytic stage, which is a requirement for secondary metabolite production. Active biomass grows in the nutrient rich zones, and the nutrient stressed biomass, which is highly productive, in terms of secondary metabolites, is in the nutrient poor zone.

Membranes suitable for the MGR system

The important feature of the polysulphone membrane was the regularity of the microvoids present in the substructure and the complete absence of an external layer. This feature allowed the microvoids to be inoculated with fungal spores or mycelial cells by reverse filtration. Membrane morphology may be classified into symmetric and asymmetric structures. The thickness of symmetric membranes (porous and nonporous) ranges roughly between 10 to 200 µm. Asymmetric membranes consist of a dense top layer (skin) with a thickness of 0.1 to 0.5 µm. These membranes combine the high selectivity of a dense membrane with the selectivity with a high permeation rate of a thin membrane (Howell et al., 1993). According to Jacobs and Sanderson (1997), capillary membrane by definition, are narrow-bore tubular-type membranes, typically with an outside diameter, which ranges from 0.4 to 2.4 mm. Unlike the large-bore tubular membrane types, capillary membranes, because of their small diameters, are self-supporting. The capillary membranes used by Leukes et al. (1999), fall in the category of integrally skinned asymmetric membranes. Integrally skinned refers to the skin layer of the membrane being an integrated part of the membrane substructure. Asymmetric refers to the graded porosity of the membranes substructure, which is most dense just below the skin layer, but increasingly porous with distance away from the skin layer. The substructure is sponge-like, and it contains finger-like microvoids (Jacobs and Leukes, 1996). Internally skinned capillary polysulphone (coded IPS 763) and ceramic membranes showed the best attachment and immobilisation of *P. chrysosporium* spores compared to tubular membranes. Average biofilm thickness across the length of the membrane exceeding 1000 µm were achieved on capillary ceramic membranes, 830 µm on capillary polysulphone membranes while 450 µm on tubular membranes. The characteristics of capillary membranes suitable for use in the MGR systems

Table 1. Characteristics of capillary membranes successfully used in the MGR system (Sheldon and Small, 2005).

Material / Parameter	Polysulphone	Ceramic Titanium oxide
Outer diameter (m)	0.0012	0.0029
Inner diameter (m)	0.0009	0.0019
Wall thickness (m)	$\pm$ 200 x 10^{-6}	0.001
External average pore size (µm)	11	3
Internal average pore size (nm)	-	0.9
Burst pressure (kPa)	1400	8000
Operating pH	4 – 11	0 – 14
Maximum operating temperature (°C)	50	350

Table 2. Comparison of *MnP* concentrations and productivity with other continuous bioreactor systems.

Reactor and processdescription	*MnP* concentration ($U.L^{-1}$)	Productivity($U.L^{-1}.day^{-1}$)	Reference
Packed bed (continuous)	250	202	Moreira et al., 1997
MGR (continuous)	2361	1916	Leukes, 1999
MGR (continuous)	15171	428	Garcin, 2002

tems are listed in Table 1. It was determined that capillary ceramic membranes, are suitable for industrial production of secondary metabolites because; 1) microbial stress in the immobilised biofilmswill be higher, due to thicker biofilm thickness, 2) were more rigid (mechanically stable) and 3) could be chemically and steam sterilized (Sheldon and Small, 2005).

Production of secondary metabolites: SCMGR vs MCMGR

The scale-up process of the MGR was performed using *P. chrysosporium* ME446. A 10-fold scale-up from a single capillary MGR to a 2.4 L MCMGR resulted in a 7-fold increase in *MnP* production with a peak enzyme activity at 209 $U.L^{-1}.d^{-1}$ (Govender et al., 2003). A 0.5 L, 15-membrane MCMGR, with nutrient capsules at the top and bottom of the reactor, was designed for continuous secondary metabolite from biofilms of *P. chrysosporium* BKMF 1767. *LiP* activity peak of 40 $U.L^{-1}.d^{-1}$ was achieved without the use of technical grade oxygen (~100%) to improve ligninolytic activity in the biofilms (Ntwampe, 2005). Even at a developmental stage, the MGR shows great potential as a secondary metabolite producing system. Comparisons of MnP concentration and productivity with continuous systems are shown in Table 2.

Growth kinetics and substrate consumption in the MGR

Research on the growth kinetics and consumption of essential nutrients in the MGR using a well-researched microbe, *P. chrysosporium* BKMF 1767 (ATCC 24725), was done using the nutrient medium of Tien and Kirk (1988) which was developed for this fungus. The nutrient medium contained 55 mM glucose and 1.1 mM ammonium tartrate as the ammonium source (Tien and Kirk, 1988). In the membrane gradostat reactor systems, in which *P. chrysosporium* was immobilised on polysulphone membranes, the logistic curve showed primary and secondary biofilm growth phases, which was later established as a phenomenon in *P. chrysosporium* biofilms, as the regeneration of mycelium occurs in successive growth cycles (Kirk et al., 1978; Yetis et al., 2000; Ceribasi and Yetis, 2001). The dry biomass generated on the membranes increased during the first 168 h (7 days), after which it stabilised until 216 h (day 9), with the secondary phase occurring thereafter. The results obtained are similar to those obtained by Kirk et al. (1978), were the secondary growth phase occurs after 10 days (mycelia cultured at 39°C, in a 125 ml flask with 21% O_2). The following growth phases were identified in the MGR biofilms: 1) lag phase (0 to 48 h); 2) exponential growth phase (72 to 120 h); 3) stationary phase (144 to 216 h); and 4) secondary growth phase (>216 h). The stationary phase was classified, where the harvested biofilms mass did not change for a period of 48 h. The dry biomass density was physically measured using a helium pycnometer. The representative growth rate constant for *P. chrysosporium* biofilms in the MGR was determined as 0.035 h^{-1} (Ntwampe, 2005; Ntwampe and Sheldon, 2006). Substrate consumption by the immobilised biofilms was determined by measuring the difference between carbon and ammonium sources in the feed and permeates reco-

vered. The increase in carbon source (glucose) consumption was determined; in different stages similar to those observed from dry density, and generated biomass during MGR operation. The rate of glucose consumption was 94.7 g/m^3.h over a period of 264 h, compared to 43 g/m^{3}h obtained in a Trickle Fixed Bed Reactor operated for a similar period of time (Ntwampe and Sheldon, 2006; Bosco et al., 1996). The basis for comparison was that both systems were continuous enzyme production systems where the nutrient medium, was that designed by Tien and Kirk (1988) and a similar microbial strain, *P. chrysosporium* BKMF 1767, was used. This showed an improved use of nutrients. The maintenance coefficient was calculated as 0.028 h^{-1}. After the lag phase (2 days), 90% of ammonia tartrate supplied daily to the MGR at a rate of 1.68 ml/h was consumed (Ntwampe, 2005). Overall, growth kinetics obtained in the MGR was similar to those determined for submerged batch cultures operated for a similar period of time.

MGR'S operational requirements

Bioreactor orientation: horizontal vs. vertical

The conditions that are optimal for biomass accumulation and development in membrane reactor systems are not necessarily the same as those for secondary metabolite production. Permeate flux due to pressure effects were evaluated on a horizontally placed bioreactor systems using deadend filtration mode. A considerable variation of flux was established in clean fibres length without biofilm attachment, as well as between different fibres of the same length (Garcin, 2002). Theoretically, horizontal bioreactors will minimise radial substrate gradients in the biofilms, due to gravitational forces acting on the nutrient medium. Consistent radial substrate distribution in the immobilised biofilms is a requirement for an efficient MGR. The biofilms produced are required to be axially homogenous across the length of the membrane and thick enough so that a nutrient gradostat across the biofilms can be attained (Leukes, 1999; Leukes et al., 1999). Vertically orientated MGR systems have an advantage that permeate will proceed down the length of the membrane and establish the required gradients. This also means more efficient use of essential nutrients. In MCMGR systems, droplet formation from other fibres will not interfere with biofilm growth on other membranes when reactors are vertically placed. Biofilms grew on reactor walls in horizontally placed MGR systems because of biofilm detachment caused by gravitational forces. This caused mycelial growth in the permeate to be recovered. However, vertically placed reactors had; 1) consistent biofilm development and thickness across the length of the membrane and 2) could be operated longer without permeate contamination. It was concluded that horizontally placed MGR systems, would not be suitable for an efficient membrane gradostat due to; 1) uneven biofilm growth on the membranes, because of flux maldistribution, 2) biofilm contamination in the recovered permeate samples, and 3) sagging of polysulphone membranes due to heavy biofilms in the bioreactor. The proposed MGR operational set setup is as shown in Figure 3.

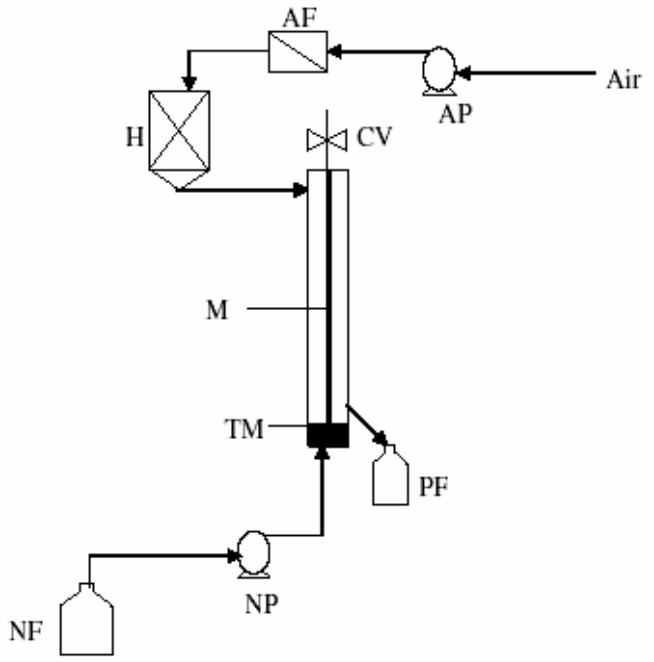

Figure 3. Schematic illustration of a vertical single capillary membrane gradostat reactor set-up.
AF: air filter; AP: air pump; CV: closed valve (dead-end filtration mode applied); H: humidifier; NF: nutrient flask; NP: nutrient pump; PF: permeate flask; M: polysulphone or ceramic capillarymembrane TM: Teflon mould. Note: The air was supplied on the shell side and it helps with the flow of permeate to the permeate flask (PF).

Nutrient and air/oxygen supply

Peristaltic pumps are generally used to maintain and to achieve low flux across the length of the membrane. Low pumping rates of the nutrient medium are used, so that appropriate radial nutrient gradients in the biofilm can be established. Liquid medium flow rates of less than 3 ml/h per 120 mm of polysulphone membranes are used for secondary metabolite production. Air/oxygen is supplied at approximately 1 vol.ECS^{-1}.min^{-1} or less, to avoid sloughing of the biofilm. The air/oxygen is passed through a humidifier into the EC using an air pump so that the aerial biomass in the MGR would not dry out. However, other operational parameters like nutrient mass transfer kinetics in the biofilms immobilised in the MGR system have not yet been determined.

Conidia (spore) inoculation and germination

The inoculum for MGR systems can be prepared by suspending conidia or mycelia in sterile distilled water. Inoculation take place by reverse filtration mode where by suspended conidia/mycelia solution is pumped through the shell side via the permeate port (Govender et al., 2004). The water passes through the external surface of the membrane to the lumen side leaving the conidia entrapped in the cavities on the externally unskinned polysulphone membrane. The backpressure can be monitored to ensure that the pressure limit (150 kPa) is not exceeded. The inoculation solution must all be passed through the system to ensure that quantified numbers of conidia, quantified using spectrophotometry techniques are immobilised. To allow for conidia germination and mycelia attachment, a small amount of nutrient medium is passed through the system and stopped for approximately 24 h. As the MGR is operated in dead-end filtration mode, this precaution prevents conidia from detaching from the cavities into the permeate flask in the initial stages of bioreactor operation (Luke and Burton, 2001). It was determined that using smaller volumes of the inoculation solution, which does not exceed twice the ECS volume of the MGR, prevents elongated inoculation procedure and increases immobilisation without compromising the integrity of the membranes or bioreactor system (Ntwampe, 2005).

Bioremediation applications using the MGR

The production of laccase using the MGR system was sustainable for a period of 30 to 40 days at 10 U/ml using the fungus *N. crassa*. Phenolic compounds, phenol and *p*-cresol, were effectively removed from 5 mM solutions, when the solutions were fed through the lumen of the MGR to the immobilised *N. crassa*. The biofilms immobilised in the MGR sustained removal efficiency continuously for a 4 month period, whereas batch cultures, remained active for 8 to 15 days; after which cultures were no longer viable (Luke and Burton, 2001). Polyphenol oxidase was immobilised on the externally unskinned polysulphone membrane and 949 µmol phenolics were removed from a solution containing 4 mM total phenolics continuously supplied to the MGR system (Edwards et al., 1999). Average phenol content from olive wastewater was reduced from 30 to 10 mg/L over a period of 24 h using permeate from a multi capillary MGR system were biofilms of *P. chrysosporium* were immobilised (Ntwampe, 2005). These applications show the versatility of the MGR system.

Commercial potential of the MGR

Synexa Life Sciences (Bellville, South Africa) currently use MGR's for the production of commercial secondary metabolites from various species of microorganisms.

The use of the MGR has showed to optimise production when compared to submerged fermentations. The design targets the biotechnology, bioprocessing and biopharmaceutical manufacturing industries. Research and development costs are relatively high in pilot plant scale and the use of submerged cultures is outdated as they are: 1) inefficient, in terms of increased product development lead-time, 2) the systems are anaerobic and 3) the bioreactors operate in high shear environment due to aeration and stirring. Currently the use of genetically modified organisms (GMO's) is on the increase, but this causes high development costs and increased allocation of human capital. The MGR is advantageous because, 1) it closely mimics the natural environment in which most microbial biofilms live, 2) allows extended continuous production of secondary metabolites, 3) it generates low waste, thus reduce waste disposal costs and 4) works well with wild type strains, thus reducing the need for genetic engineering.

Concluding remarks

The use of the MGR or other membrane reactor technology systems in the biotechnology and biopharmaceutical production of secondary metabolites is limited. Much of the membrane bioreactors are currently used in the wastewater treatment industries. The concept of a membrane gradostat, have been addressed and discussed in this manuscript. Capillary membranes suitable for use in the MGR and the characteristics are listed. The membranes have an external unskinned porous structure, with an internal skin. The membrane structure was previously determined to be suitable for biofilm attachment. Operational conditions are highlighted, which include the choice to operating the MGR vertically. The use of multicapillary membranes for the production of secondary metabolites was compared to the single capillary MGR systems. The versatility of the MGR systems and its commercial and industrial potential were highlighted.

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