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THE CONCEPT AND PRACTICAL CHALLENGES OF MEMBRANE GRADOSTAT REACTORS

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Developing a new technology from a theoretical concept to its industrial application is generally a long and burdensome procedure. The membrane gradostat reactor (MGR) – which was invented for the continuous production of secondary metabolites – is now at the initial stage of this process. Therefore, even though it was patented a quarter of a century ago, relatively few studies have been published concerning this device. In this opinion article, the structure of the MGR, transport mechanisms through the biofilm and membrane, methods and challenges of inoculation as well as the aspects determining the structure of a suitable membrane and species will be overviewed. The findings are based on the observations from other publications as well as the own experiences of the authors.

Keywords: membrane bioreactor, secondary metabolites, biofilm structure, *Streptomyces*

1. Introduction

Microbial secondary metabolites are extremely important in the life of microorganisms as well as in the human economy. Besides antibiotics and other pharmaceutical compounds, their lesser-known representatives include biosurfactants, food additives, environmentally-safe herbicides and insecticides [1]. Among the microorganisms that produce them, *Streptomyces* species are of great significance as they are the basis of around 100,000 antibiotics and a wide range of additional bioactive compounds such as pesticides, neurogicals, enzyme inhibitors and siderophores [2].

In compliance with their biological roles – e.g. protection against other species or toxic conditions, decomposition of complex nutrients, etc. – secondary metabolites are produced in the late or stationary growth phase when unfavorable conditions evolve such as the exhaustion of nutrients [1]. This environmental signal triggers a global alteration in gene expression resulting in morphological differentiation as well as a shift towards secondary metabolism. While stress factors are necessary for production, they hinder bacterial growth and cannot be maintained in the long term [3]. Therefore, batch operations have proliferated in the industry in spite of the advantages of a continuous operation, e.g. higher throughput, advanced quality control and economic benefits [4].

This issue could be handled by a unique device called a membrane gradostat reactor (MGR), a biofilm reactor which enables the continuous renewal of biofilms and the production of secondary metabolites simultaneously. Due to the construction of the reactor, downstream processes have become simpler than in the case of conventional submerged fermentations, therefore, the effluent does not contain large amounts of biomass. Moreover, the extracellular polymeric substances (EPS) fraction of the biofilm – a valuable source of biomaterials applied in the pharmaceutical, food and cosmetics industries [5] – can be harvested after fermentation cut-off.

While these advantages make MGRs a promising technology, only a limited number of publications have been written concerning these devices since they were patented in 1999 [6]. Therefore, several questions remain unanswered regarding both their theoretical foundation and practical implementation. From among these issues, this study focuses mainly on the inoculation process as well as the requisite properties of the membranes and microorganisms which make them applicable in MGRs. Regarding species, the *Streptomyces* genus is focused on because of its industrial importance.

Besides the main findings of other publications, this article presents the own experiences of the authors, which did not lead to a quantified result but can help to understand and develop the system. Due to limited space, details of these experiments – all of them carried out with *Streptomyces coelicolor* – are not presented, however, they are provided as supplementary data. The aim is to

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draw attention to this system and its untapped potential while revealing a part of the long and winding road that hopefully will lead to the successful implementation of this technology.

2. The structure of an MGR

An MGR is a bioreactor in which capillary membranes serve as a solid support for the biofilm and maintain a nutrient gradient inside it. When operating, the medium solution is fed into the lumen of the hollow fibres from which the nutrients diffuse through the membrane onto its surface where microorganisms form a biofilm. Since the hollow fibres are surrounded by air, the oxygen diffuses through the biofilm in the opposite direction to the components of the medium [7].

As the biofilm – along with growth of the biomass – thickens, different zones evolve inside of it according to substrate availability (*Figure 1*). The layer of the biofilm closest to the membrane gains enough nutrients to grow and reproduce [7], while further away from the membrane, i.e. from the nutrient source, the cells are in the stationary or declining phase and nutrient limitation leads to the production of secondary metabolites.

In conclusion, the special structure of an MGR makes the system suitable for the production of secondary metabolites in the long term due to continuous biofilm renewal. Moreover, culture conditions in the MGR are similar to in the natural environment of soil bacteria like *Streptomyces* and several other microorganisms [8]. This feature might be favorable in the industry, especially in the case of those species that are sensitive to shear stress [9] or cannot produce secondary metabolites during submerged fermentation [10]. However, certain aspects of this system have not yet been comprehensively researched. Will the mentioned zones really evolve or the effective nutrient transport inside the colonies hinder the development of a gradient? What happens to dead cells in the outermost layer? Will they be removed (as in the case of submerged cultures) or remain attached resulting in an "infinitely" growing biofilm? What are the limitations of this process? In the following sections, some of these issues will be addressed.

3. Gradients and transport in the biofilm

3.1. Limitations of biofilm thickness

Based on the theoretical concept of MGRs, biofilm thickness presumably has a lower boundary which has to be reached in order to achieve nutrient limitation in the outer layer. Our assumption is that the value of this boundary might depend on the species and fermentation parameters such as the temperature, pH and composition of the culture medium. This is based on the observation that the listed parameters have a great impact on the properties of EPS that strongly influence biofilm formation and the transport process through the biofilm

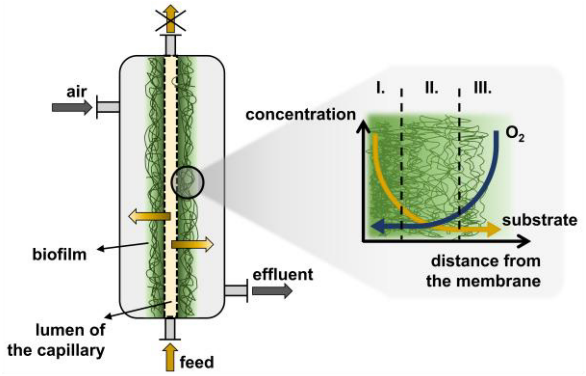

Figure 1: Structure and concept of a single capillary MGR (The phases consist of primary growth (I.), stationary growth (II.) and decline (III.)) (This figure was created using Figure 2 of [7])

[11],[12]. However, to the best of our knowledge, no studies have been published focusing on this issue, and thus these hypotheses cannot be confirmed.

On the other hand, since air is supplied in the extracapillary space, the direction of the oxygen gradient is the opposite to that of the nutrient gradient. As a result, the outer layer of the biofilm gains the most oxygen while the inner regenerating layer gains less. As oxygen penetration is limited, an upper boundary thickness, presumably depending on the species and other parameters, similar to that of the lower boundary might result. Above this value, oxygen deficiency might occur in certain zones which might cause severe problems in the case of aerobic microorganisms. These regions become inactive and separate from the membrane resulting in the leakage of culture medium and the absence of a nutrient gradient [9].

In order to prevent an oxygen shortage, different strategies have been applied during the cultivation of *Aspergillus terreus* in a single capillary MGR [9],[13]. One of them is controlling the biofilm thickness by a so-called two-stage feeding strategy [9]. After inoculation, a culture medium optimal for cell growth is provided until the desired biofilm thickness is achieved. At that point, further growth might be hindered by the changing composition of the medium since cells enter the stationary phase and start to produce secondary metabolites. This effect might come about by altering either the nitrogen source [13] or C/N ratio of the culture medium [9].

Another possible approach concerns the influence of the structure of the biofilm. It has been observed during microparticle-enhanced cultivations (MPEC) that cell morphology might be affected by the addition of microparticles [14]. Based on this, Mohseny et al. [13] supplied talc particles (hydrous magnesium silicate) to the spore suspension which led to a looser structure and improved oxygen penetration even though the biofilm thickness was approximately the same as in the case of the control.

3.2. Effect of biofilm growth on the hydrodynamics of MGR

Biofilm growth affects not only oxygen uptake but also the transmembrane flux of a culture medium. A study by Godongwana et al. [15] on the hydrodynamics of MGR is summarized as follows:

A single ceramic capillary membrane was inoculated with *Streptomyces coelicolor* and introduced into the MGR in the dead-end mode. The differential pressure across the membrane was constantly maintained at 3 kPa, moreover, the biofilm thickness and volume of the permeate were measured in order to calculate the flux and hydraulic permeability (neglecting fouling). Over the first 72 hours, as the biofilm was growing, the transmembrane flux and consequently the hydraulic permeability decreased as expected. However, after this period, the permeability remained approximately constant and fluctuated only slightly while the biofilm continued to thicken by about 2.5 times.

These results are explained by the special structure of the biofilm. Starting from the surface of the membrane, three different regions could be detected in the biofilm, namely the primary growth zone, transition zone and aerial mycelium zone. Based on the previous findings by the authors, the primary and aerial mycelium layers grow only in the first period – in this case, during the first 72 hours – therefore, further increase of the biofilm thickness depends on the development of the transition zone. Hence the primary zone mainly determines the density of the biofilm, while the hydraulic permeability remains unchanged after the thickness of the primary layer becomes constant.

4. Inoculation of the MGR

The generally applied method of inoculating the MGR is reverse filtration. The name denotes that the inoculum is filtrated through the membrane from the ECS to the lumen so the flow direction is opposite to that of the nutrients. The inoculum is pumped into the shell side and the ECS is pressurized in order to channel the inoculum towards the hollow fibers. The liquid phase passes through the membrane but spores are retained which attach to the membrane surface or the walls of the pores [16]. During the initial period of reactor operation (approximately the first 24 hours), it is advisable to fill the lumen with the nutrient medium and close all inlets and outlets of the system. In this way, spores can germinate and strengthen their affinity with the membrane, thereby avoiding the washing out of spores in the nutrient flow [17].

Govender et al. [16] assumed – based on observations by previous researchers – that using vegetative mycelium instead of spores as an inoculum might lead to inconsistencies in the biofilm. However, according to a recently published study investigating the effect of the harvesting time and pH on the filtration characteristics of the fermentation broth [18], the features of broths can be significantly influenced by the aforementioned parameters, therefore, it is possible that optimal conditions for inoculation might be reached so this is worthy of further investigation.

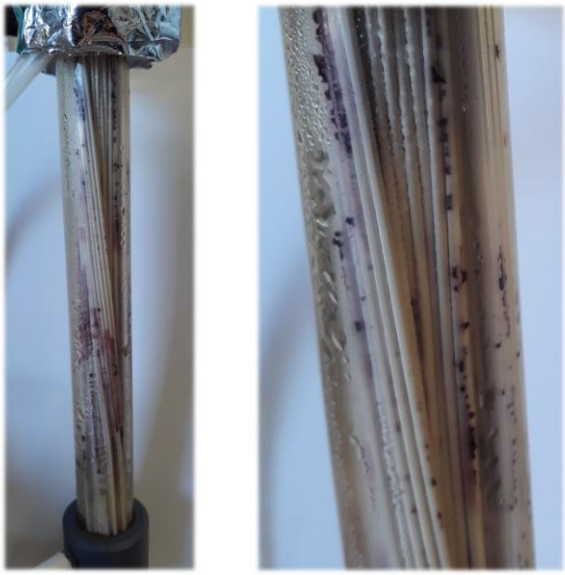

Figure 2: Incontiguous colonies on the surface of the Pentair X-Flow hollow fibre module

5. Microorganisms

During an optimal inoculation method, spores or cells are evenly distributed on the membrane surface. Even if they do not cover the whole surface, motile microorganisms can colonize the membrane during cell growth resulting in a homogeneous biofilm. The genus *Streptomyces*, however, has evolved to maximize nutrient uptake within a certain region and protect themselves by inhibiting neighboring microorganisms [19], rendering them one of the greatest producers of secondary metabolites, although their ability to colonize further areas is strongly limited. In this way, they do not tend to spread on the membrane surface in MGRs.

This is neatly illustrated by the experiment with a Pentair X-Flow ultrafiltration membrane module conducted by the authors, which was inoculated with a spore suspension of *Streptomyces coelicolor* by reverse filtration. During the inoculation, spores only attached to the membrane in certain regions probably due to unfavorable flow conditions. As the experiment proceeded, cells did not colonize the membrane surface but remained at their starting point and passed into the secondary metabolite-producing phase. The results can be easily visualized because the microorganisms produce pigmented antibiotics so their colonies are deep purple in color (*Figure 2*).

Two recently discovered phenomena might enable this problem to be resolved.

It is already known in nature that insects and nematodes spread *Streptomyces* spores, however, is has only recently come to light that motile microbes are capable of transporting their immotile counterparts over

short distances [20]. Mouk et al. [21] observed that *Streptomyces coelicolor* spores can attach to the flagellum of *Bacillus subtilis*, thereby taking advantage of its motility. They use the apt 'hitchhiking' term to describe this type of movement and suggest that it is conserved across the genus *Streptomyces*.

Another novel discovery is the special exploratory behavior of *Streptomyces* species, which occurs in response to certain environmental conditions. The exact underlying mechanism of such behavior is not fully known but is presumably triggered by the glucose consumption of neighbouring yeast. In addition, a rise in pH and the presence of certain volatile organic compounds (e.g. trimethylamine) may also play a crucial role in this process. Although explorer cells are hydrophilic just as 'normal' vegetative hyphae, they are nonbranching, which may enable these colonies to spread, even though abiotic surfaces. Moreover, some species produce surfactants to further facilitate exploration [19].

The aforementioned phenomena might be exploited to facilitate membrane colonization in MGRs by cocultivation. For this purpose, such an 'auxiliary' species has to be found that supports the development of a suitable biofilm but that does not impair the growth nor secondary metabolite production of the producer species. Since exploratory growth might be triggered by applying VOCs as info-chemicals, the presence of another microorganism might be unnecessary.

On the other hand, another aspect other than the colonizing ability has to be taken into consideration when choosing a species that is suitable for inoculation, namely intracellular transport and its effect on substrate distribution in the biofilm.

Regarding fermentations in MGRs, filamentous organisms are predominantly used as producing strains, e.g. filamentous fungi [9],[22] or actinomycetes [8],[15]. There are a number of reasons for this choice: the wide range of important extracellular secondary metabolites [1], the proven ability of forming a biofilm on their own [23],[24] as well as the fact that the filaments penetrate the pores before becoming interwoven, therefore, adhesion between the membrane and biofilm mat is expected to be stable. However, filamentous microorganisms exhibit apical growth supported by intracellular transport, a phenomenon which is barely taken into consideration when it comes to the development of radial gradients in MGRs.

The fungal hyphae elongate apically. The filamentous growth of fungi is facilitated by effective intracellular vesicle transport to the hyphal tips by mechanisms that require microtubules and an F-actin cytoskeleton [25]. The apical growth of *Streptomycetes* by elongation resembles that of filamentous fungi which implies the existence of inner transport mechanisms. However, these transport mechanisms are less sophisticated compared to fungi as they are prokaryotes. In the absence of a complex cytoskeleton, they rely primarily on diffusion [26], which might be limited by the cross-walls [27], therefore, the chance of a gradient forming might be higher than in the case of fungi.

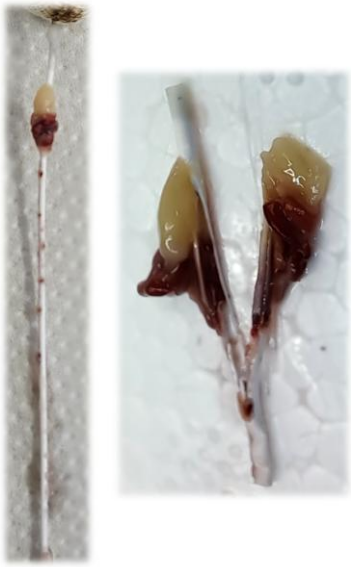

Figure 3: Biofouling inside the hollow fibre

Based on these findings, investigating the application of unicellular microorganisms would be worthwhile as in their case the development of a radial gradient is more likely than by filamentous microbes.

6. Membrane

As it was previously mentioned, the membrane plays a pivotal role in an MGR by providing a solid surface for the cells to grow on and enabling the maintenance of a nutrient gradient inside the biofilm. Accordingly, the membrane is expected to fulfil several criteria, most importantly:

- surface properties (e.g. hydrophobicity, rugosity, surface area, etc.) favorable for cell adhesion [16];
- high porosity, preferably with increasing pore size towards the outer surface;
- sterilization capability (either chemically or with steam);
- mechanical stability (especially important during the long-term operation of large-scale MGRs).

In most research, polysulfone or ceramic membranes were applied, which exhibit certain different qualities that should be taken into consideration when choosing a suitable membrane [7]. It is worth mentioning that commercially available membranes are generally designed to avoid biofouling, which is important for most applications (like in microbial fuel cells [28]), however, antifouling is a hindrance in the case of MGRs. These membranes have a relatively small surface area with blunt-ended macrovoids that hinder cell attachment, moreover, an external skin is also often applied to further enhance this feature [29]. In contrast, the hollow fibers applied in MGRs are microfiltration membranes with a sponge-like structure and cavities on the outer surface provide a large surface area for cell attachment. On the lumen side, an ultrafiltration skin layer is formed in order

to prevent cells getting into the lumen [7]. Although this precaution might seem excessive, it might be essential in some cases as exemplified by the following observation by the authors.

During experiments with a single capillary MGR, it was experienced in some cases that on the upper region of the membrane – close to the feed – the biofilm developed as a clump, while below this cluster, colonies were only sparsely present. After cutting open the membrane, it was ascertained that the microorganisms passed through the wall of the membrane, colonized its inner surface and clogged the lumen, therefore, the lower region had no access to a nutrient medium and cell growth was hindered (*Figure 3*).

There are two possible explanations for this phenomenon. While hollow fiber membranes can be characterized by a mean pore size, a few pores with much larger diameters might develop during the production process. When applied in non-biological systems, large pores might slightly reduce selectivity but not significantly. However, in bioreactors, even if only a few cells get across the membrane, they can grow and clog the capillary, thereby hindering the flow of the culture medium.

Assuming the absence of such large pores, *Streptomyces* species have a surprising ability, that is, they can pass through membrane pores much smaller than the diameter of hyphae. Observations by Wolf et al. [30] indicate that these microbes are flexible to a certain extent – the diameter of their hyphae can shrink from 0.7 to 0.2 μm – allowing them to grow through conventional microfiltration membranes.

Based on these experiences, an inner ultrafiltration layer is a requisite for membranes in MGRs in order to prevent cell infiltration into the lumen – at least in the case of *Streptomyces* species.

7. Conclusions and perspectives

Theoretically, membrane gradostat reactors might be suitable for the production of any microbial secondary metabolites during continuous operation. However, as was pointed out in this article, some practical issues may limit the scope of potential producing microorganisms. Among other factors, transport mechanisms inside the biofilm and the colonizing ability of the microorganism should be scrutinized when choosing the species. Furthermore, since commercially available membranes are mostly designed to exhibit antifouling properties, the modification of existing membranes or the creation of new ones might be necessary. Developing alternative inoculation methods is also worth considering as they strongly affect the efficiency of MGRs.

Should the gradostat concept not be feasible – due to the structure of the biofilm or the transport mechanisms inside it – the system still exhibits several beneficial properties such as:

- culture conditions mimic the natural habitat of many microorganisms which might have a positive impact on secondary metabolite production;
- product recovery and downstream processes are relatively simple;
- the EPS fraction of the biofilm can be easily harvested after fermentation cut-off.

In order to make this technology available on an industrial scale, several theoretical and practical issues should be addressed. Future lines of research should focus on the feasibility of the gradostat concept; the development, structure and ageing of the biofilm; the practical limitations of the system; as well as the methods of inoculation and process control.

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