Review

Biofilms as living catalysts in continuous chemical syntheses

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Biofilms are resilient to a wide variety of environmental stresses. This inherited robustness has been exploited mainly for bioremediation. With a better understanding of their physiology, the application of these living catalysts has been extended to the production of bulk and fine chemicals as well as towards biofuels, biohydrogen, and electricity production in microbial fuel cells. Numerous challenges call for novel solutions and concepts of analytics, biofilm reactor design, product recovery, and scale-up strategies. In this review, we highlight recent advancements in spatiotemporal biofilm characterization and new biofilm reactor developments for the production of value-added fine chemicals as well as current challenges and future scenarios.

Biofilms as living biocatalysts

In nature, the microbial lifestyle is dominated by biofilms. The term 'biofilm' refers to surface-associated microbial communities as well as microbes forming flocks and aggregates. Biofilm organisms develop on all kinds of interfaces (e.g., oil/water/air) and are embedded in self-produced extracellular polymeric substances (EPS) in which they live in a coordinate fashion, thereby benefitting from ecological niches formed within the biofilm (for more details on biofilm development, see Box 1). The properties of these microbial societies are governed partially by the structure, diffusion, and physiological activity of the community [1]. Biofilm-growing organisms are self-regenerating, spatially and metabolically well organized, and are in general less affected by toxic substrates and/or products. Their overall robustness makes them attractive living catalysts for organic syntheses, which can be harnessed for technical applications. At the nexus of ecology and engineering, we describe biofilms as living biocatalysts for performing challenging conversions in controlled environments.

Biocatalysts are isolated enzymes or whole living cells. Application of whole cells as biocatalysts is commercially well established and often preferred over isolated enzymes. The prime reason is to gain catalyst stability, self-generated expensive cofactors, and reactions involving multicomponent assembly of membrane-bound proteins [2]. In addition, enzyme purification often adds significantly to the overall production costs, posing a strong argument for using whole cell catalysts. Whole cell processes are so far mainly confined to batch and fed-batch processes in which microbial cells are often grown in suspended mode and are not reused. This might be due to substrate and/or product toxicity, short-term biocatalyst stability, biocatalyst retention, catalyst regeneration, and the complexity of running a large-scale chemostat. However, continuous reactor operation is cost-effective because of reduced downtimes for reactor preparation, cell growth, and cleaning. In

Glossary

Atomic force microscopy (AFM): technique for generating images of surfaces by measuring the physical interaction between a sharp tip and the sample specimen.
Biocatalysis: transformation of a chemical substance into a desired product by an enzyme or whole cell (biotransformation) or transformation of a substance that also serves as a growth substrate into a desired product by a whole cell biocatalyst (fermentation).
Confocal laser scanning microscopy (CLSM): technique for generating 3D images of biofilms. Confocal microscopy uses high excitation energy provided by laser lines to excite fluorophores originating either from externally added fluorescent dyes or from intracellular fluorescent proteins. The excited fluorescence is detected by photomultiplier tubes. The biofilm specimens are scanned with high penetration depths (>100 μm) to produce serial optical sections or stacks, which are then reconstructed to obtain 3D images of the native biofilm structure at the micron scale.
***In situ* product extraction**: techniques applied to remove the product immediately as it is formed in the vicinity of the microbial cell during a running process.
Microbial-assisted electrosynthesis: microbially catalyzed synthesis of chemical compounds in an electrochemical cell.
Microsensor: a tool used to monitor microbial activities, as well as physical and chemical gradients *in situ*. Based on the working principle and construction, these sensors are classified as electrochemical sensors (microelectrodes, biomicrosensors) and fiber-optic microsensors (microprobes or micro-optodes). Electrochemical sensors function based on electrochemical reactions, e.g., Clark type oxygen microelectrode. Fiber-optic microsensors are based on either irradiance or fluorescence light and thereby take advantage of the fiber-optic light guiding capacity. Most microsensors have a tip size diameter of 1–100 μm, which allows for a high resolution (≤0.1 mm).
Optical coherence tomography (OCT): technique for obtaining mesoscale structural details and images of biofilms. OCT uses the optical back-scattering of light to rapidly scan images. Through the use of near infrared wavelengths, image penetration is in the millimeter range.
Quorum sensing (QS): cell-to-cell communication mechanism of bacteria to respond to and monitor their population density.
Scanning electron microscopy (SEM): technique to visualize surface structures. A finely focused high energy electron beam is used to scan the sample specimen. The secondary and back-scattered electrons are captured to obtain high resolution images. Vacuum is applied to avoid high scattering of electrons in air.
Two-liquid phase concept: this concept prevents toxic effects of potentially interesting substrates and/or products on the biocatalyst. It consists of an aqueous phase for cell growth and an organic water immiscible solvent. The organic solvent acts as a sink for substrate and product and concomitantly regulates the concentration of both in the aqueous phase according to the partition coefficient of the respective substances. This avoids substrate and/or product inhibition, while the equilibrium of the desired reactions can be guided in the direction of the product.
Viable but not culturable state (VBNC state): this term refers to cells that neither grow nor die (dormant, quiescence), similar to persister cells. The VBNC state is induced by various types of chemical and environmental stress factors.

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Box 1. Biofilm development in process engineering

Which microbes form biofilms? Which surfaces are suitable for biofilm growth and which environmental conditions suit biofilm development? These are questions that arise when designing biofilm-based processes. The biofilm attachment process is very complex and will be affected greatly by cellular, surface-related, and environmental factors. Biofilm formation involves different physiological states of the respective organisms [1], which are schematically depicted in Figure I and described here. In step I, the respective substratum is conditioned by inorganic and organic macromolecules, thereby providing a nutritious zone for cell settlement. Subsequently, the interaction of the planktonic cells with the surface is started. During step II, cells attach (reversible and irreversible) to the surface via flagellar motility, surface translocation, twitching, gliding, and sliding. Following irreversible attachment to the surface, the organisms start to reproduce (step III). In addition, they secrete EPS, which attaches the cells to the surface and to each other. It consists primarily of polysaccharides, proteins, and lipids, whereas humic acids, uronic acids, and nucleic acids are present in minor amounts [8]. The EPS is crucial for the 3D biofilm structure and stability, and strongly depends on the presence of multivalent cations such as Ca^{2+}. While providing protection from the environment, excess EPS production will hinder reactor performance in a technical application and should therefore be controlled to a rather low level. In the course of biofilm development (step IV), a complex 3D architecture with channels and pores is built. These channels allow water and nutrients to diffuse through the deep layers of the biofilm, as well as waste substances to be excreted. A pseudo steady state is established in which the mature biofilm shows a dynamic turnover involving detachment of cells from the biofilm and re-growth, thus keeping the biofilm thickness stable. Surface properties such as geometry, roughness, and surface chemistry can affect the biofilm formation [70]. Finally, environmental factors such as nutrient availability and hydrodynamic conditions will affect biofilm growth and development [1]. Because the biofilm formation is governed by multiple factors, an in-depth characterization of the biofilm system is necessary to match a suitable substratum, microbial host, and process conditions for a target application. In this respect, biofilm-based process development is very time-consuming because every new strain requires a thorough characterization. As proposed by Rosche *et al.*, selection of a few well characterized biofilm workhorses would ease this process step significantly [4].

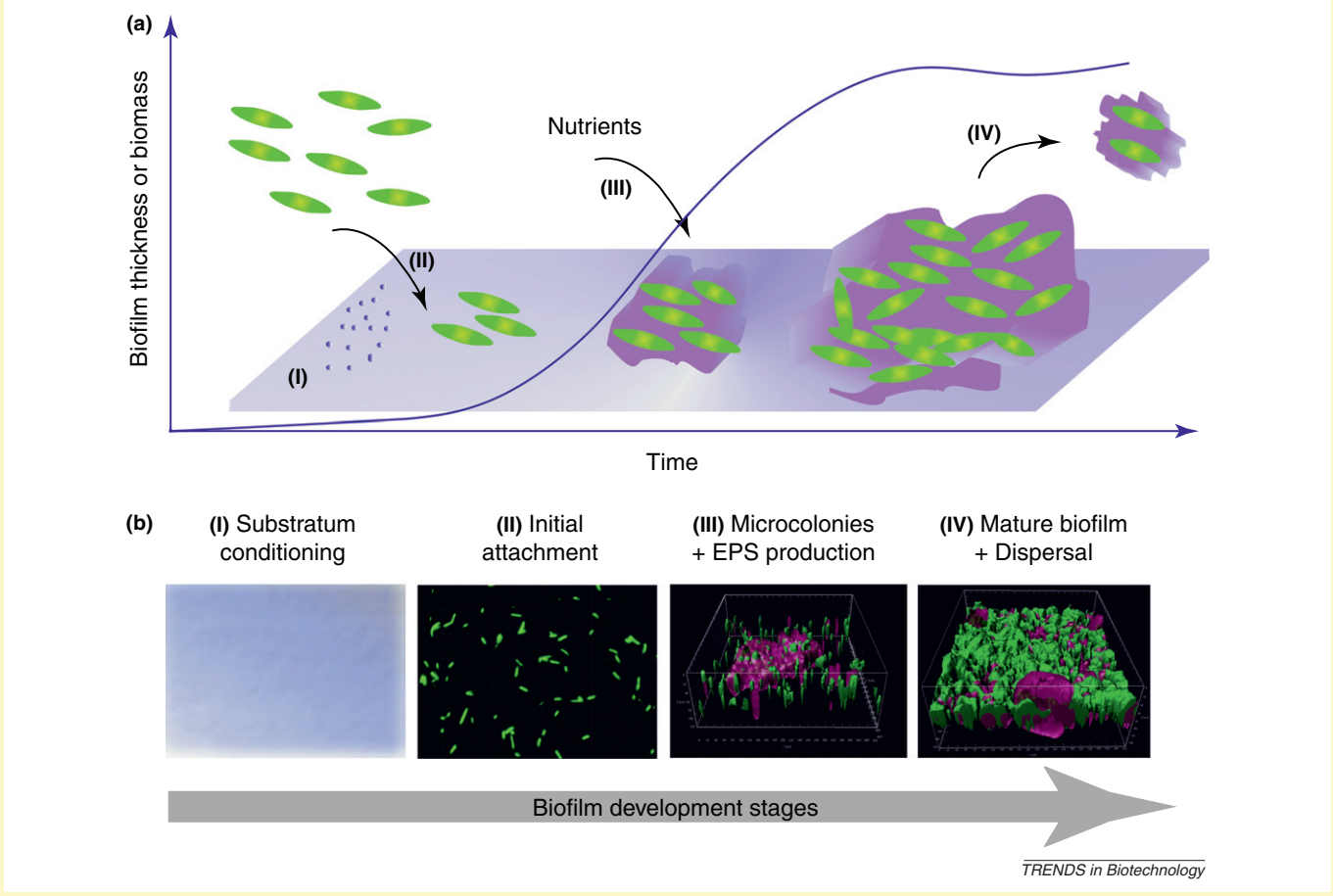

Figure I. **(a)** Simplified model of biofilm development. **(b)** Representative confocal micrographs of various *Pseudomonas* sp. biofilm development stages adapted from [17]. The green color represents the intact *gfp*-expressing cells, and the violet color represents ConcanavalinA-stained polysaccharide compounds in the EPS matrix.

addition, continuous processes offer steady state conditions with constant product qualities. Despite these features, reported industrial whole-cell continuous processes are rare for the above reasons [3,4].

One of the key methods for cell retention, reuse, and biocatalyst stability improvement is immobilization [5]. It is performed either by chemically binding cells to carrier materials or physically retaining them by entrapment or encapsulation. For whole cells, physical entrapment in a polymeric matrix is the most preferred technique whereas chemical binding is rather used for enzymes. The main disadvantages of immobilization are (i) reduced absolute activity and viability of the biocatalyst, (ii) diffusion mass transfer limitations of substrates and oxygen lowering reaction rates, (iii) additional costs, and (iv) lack of universally applicable immobilization concepts [6,7].

Naturally immobilized microbial biofilms are a solution for continuous bioprocesses. It is an elegant, powerful, and cheap method of cell immobilization, without the necessity of any added polymers or chemicals. Despite these features, utilizing biofilms as living catalysts is not yet a broadly implemented concept in bioprocess development. Here, we review the recent developments in biofilm-based productive biocatalysis (see Glossary) and techniques employed in characterizing surface-associated catalytic biofilms, discuss existing bottlenecks, and provide ideas to bridge the gap between fundamental research and industrial application.

Biofilms: a 'natural predominant microbial lifestyle' in biotechnology

In a reversible interaction with an interface, microorganisms attach and produce extracellular polymeric substances that cause a sticky framework keeping the cells together [8] (details in Box 1). Biofilm-grown cells exhibit distinct phenotypes with respect to gene transcription and growth rate [1]. Their metabolic activities are governed by the conditions at an interface and the biofilm mode of growth. Biofilm-growing organisms profit from metabolic cooperation, specialization, and a high level of structural organization among the neighboring cells. The metabolic activities of organisms growing within biofilms will lead to local variations in the biofilm environment. Different concentrations of nutrients, waste products, and signaling molecules inside the biofilm result in various niches. The organisms in this microbial community react to these changes by either varying gene expression patterns or physiological activities, thereby adapting to their local surroundings. This partially explains their increased robustness [1,9] in technical settings such as wastewater treatment plants [10], bioremediation [11], and biocatalysis [4], as well as in emerging new sectors such as fuel cells for power generation [12], which opens up a new chapter in biofilm research.

Status quo

Why are biofilms important for catalysis?

Enhanced tolerance of bacterial biofilms towards various adverse environmental conditions is a unique property that is based on various factors such as production of an extracellular matrix (EPS), different genetic regulation mechanisms mediated by quorum sensing, formation of subpopulations of dormant state VBNC cells, and metabolic and phenotypic heterogeneity of the respective organisms [9,13]. Bacterial cells in biofilms are more resistant to antimicrobial substances [14], heavy metals [15], and toxic chemicals [16,17]. These tolerance mechanisms are one of the most important features of biofilms regarding their biotechnological potential. However, they also pose major hurdles, which need to be overcome in order to implement this concept in industry. Massive production of EPS, often triggered by various stress factors [16,17] combined with extensive biomass formation impairs mass and heat transfer and leads to blocking of reactor systems. In addition, the formation of subpopulations in the VBNC state (dormant, metabolically inactive) results in a high amount of unproductive biomass, and finally in low specific catalytic activities [18]. An ideal biofilm catalyst should form a good biofilm on any given substratum of interest with a sufficient amount of EPS allowing for high biofilm robustness on the one hand and low mass transfer limitations on the other hand. Biofilm-grown cells should be genetically stable (low level of phenotypic variations, loss of plasmid in the case of recombinant strains). A genetic engineering toolbox ideally based on sequenced genomes is necessary for genetic manipulation on a genome level. The biofilm should be highly porous for the diffusion of the growth substrate, biotransformation substrates, and products. It should attain a pseudo steady state quickly to compromise the competition of growth and catalysis, while sustaining diverse adverse environmental conditions (e.g., toxicity, shear forces) with high catalytic activity. Despite these requirements, there is a growing interest in recent years in exploiting these living robust catalysts for challenging applications to produce value-added chemicals [19–21]. Biofilm-grown *Zymomonas* cells showed a much higher tolerance against the toxic benzaldehyde compared with planktonic cultures [21], whereas for the conversion of the highly toxic styrene to styrene oxide a biofilm process was reported, which ran continuously >60 days, compared to 50 hours for planktonic cultures [18]. These studies have shown that the use of biofilms may overcome major bottlenecks in classical biocatalysis, such as substrate/product toxicity, short-term biocatalytic stability, and biocatalyst retention. However, to implement biofilms on a broad basis it is necessary to overcome the above described hurdles. Significant progress was made in understanding the process of biofilm formation and dispersal on a molecular level. This knowledge opens the possibility to control biofilm growth (for details, see recent review [22]). Next to the natural molecular control mechanisms for biofilm formation, application in industry demands biotechnological solutions for reaction and process design (Box 3).

What is necessary for biofilm analysis?

This section describes a universal toolbox for biofilm investigation focusing on process relevant parameters. These include (i) spatial distribution of cells on the substratum, (ii) distribution of metabolically-active cells throughout the biofilm, (iii) analysis of the EPS matrix, (iv) biofilm thickness, (v) requirement of oxygen, growth substrates, as well as biotransformation substrates and their respective concentration profiles in the biofilm, and (vi) response to process relevant stress (e.g., solvents, shear forces). A wide array of methods for microscale investigations has been developed ranging from traditional colony forming unit (CFU) counting to intrinsically-tagged fluorescent proteins or reporter gene-coupled epifluorescence (Table 1). A good method should allow for real-time analysis and at best reflect real-process conditions of interest.

Scanning electron microscopy (SEM) and environmental scanning electron microscopy (ESEM)

Microscopic techniques are a key tool for biofilm investigation. SEM allows high resolution images for visualizing the structure of a biofilm and cells on a near-nanometer and sub-micron scale [23]. The downside of this method is that it shows artifacts due to drying of the sample. By contrast,

Table 1. Relevant techniques for catalytic biofilm analysis and their main features

Technique	Acquired information	Advantages	Disadvantages	Refs
Targets: biofilm components, properties, and microbial activity				
ATP method	Total biomass and cell numbers; biofilm growth and activity	Simplicity; cheap	Analysis dependent mostly on destructive biofilm sampling	[74]
Optical microscopy	Biofilm thickness	Simplicity; relatively cheap	Limited to biofilms with flat surface; low accuracy	[74]
Electrochemical method	Biofilm thickness	Simplicity; cheap	Not possible at initial growth stage	[75]
Uniaxial compression measurement; low load compression testing	Mechanical stability; biofilm thickness	Non-destructive and low cost; no thickness limitation; highly reproducible; cheap	Single parameter measurement	[76,77]
Chemical biofilm compounds analysis	EPS matrix composition	Relatively easy and cheap; well established methods	High standard deviation; destructive; a lot of material required	[78]
Raman microscopy	Biofilm constituents	Non-destructive and simple; spatial resolution of an optical microscope	Limited sensitivity requiring surface enhancement; not applicable on all materials; expensive equipment	[79]
Targets: biofilm structure and architecture				
OCT	Global biofilm structure at millimeter scale	Non-destructive; high resolution; reliable; relatively low cost	Should be combined with either Raman microscopy or CLSM	[35]
AFM	Topographical and morphological biofilm analysis	Non-invasive; qualitative and quantitative	Sample damage by the tip; relatively long imaging time; expensive equipment	[33,34]
SEM	Biofilm structure	High resolution	Artifacts due to drying of the sample; expensive equipment	[26]
ESEM	Sub-micron biofilm structure; EPS matrix	High resolution; possibility for examination of partially hydrated sample	Contrast effect caused by condensed water; expensive equipment	[25,26]
CLSM	Spatial distribution of cells; biofilm structure and physiology	Optical sectioning; 3D imaging; time lapse biofilm development; non-destructive	Photobleaching occurs in every method, which requires fluorescent stains; fluorescent dyes or protein labels necessary; expensive equipment	[23,27,35]
Fluorescence probing in combination with CLSM				
SYTO 9 in combination with propidium iodide	Cell vitality; membrane integrity	Simple and fast; commercially available	Needs to be calibrated for the individual strain and thus not applicable for mixed species biofilms; unspecific binding	[27]
Fluorescent reporter genes, e.g., *gfp*	Growth; activity; cell vitality; localized gene expression levels	Constitutive fluorescence; no negative side effects from external dyes	Requirement of genetically-engineered organisms	[14,17,27]
CTC assay (5-cyano-2,3 ditolyl-tetrazolium chloride)	Localization of actively respiring bacteria; spatial and temporal heterogeneity	Minimal sample preparation	Only respiratory activity is detected; not applicable for anaerobic bacteria	[9]
Fluorescein isothiocyanate; Concanavalin A; Calcofluor white; and others	EPS components; sorption properties	Low incubation time	Penetration throughout the biofilm; non-specific binding; multiple laser lines for sequential scanning necessary	[30]
Fluorescent *in situ* hybridization (FISH)	Phylogenetic bacterial identity; ribosomal activity	Precise localization of the cells in the biofilm; mapping of complex biofilm communities; also detects cells in the VBNC state	Less effective for low abundance RNAs without amplification; sometimes involves destructive sample preparation	[9,27]
Targets: biofilm processes and physiology				
Microsensors	O_2; H_2; H_2S; CO_2; Ca^{2+}; NO_2^-; NO_x; Fe^{2+}; glucose; CH_4; temperature; pH; uptake rates; diffusion coefficients	Fast and specific response to analyte; most of the sensors are commercially available at moderate costs	Fragile; time-consuming preparation; calibration necessary; slightly invasive; point measurement	[36,37]

ESEM functions in a gaseous atmosphere allowing for the imaging of wet systems with minimal specimen preparation [24–26]. However, contrast effects caused by condensed water are still a drawback. This is partially overcome by slight fixation and dehydration [25]. The EPS matrix of *Pseudomonas aeruginosa* biofilms has been mapped by introducing staining methods into ESEM analysis. These improvements enhanced the contrast of the microbial structures and easy discrimination of the EPS matrix, which is important when evaluating the

influence of EPS on the mass transfer abilities of the biofilm [26]. Yet, time-resolved online and non-destructive biofilm visualization by ESEM is still not possible, which is essential to investigate and understand biofilm structure and physiology in its natural condition.

Confocal laser scanning microscopy (CLSM)

CLSM is regarded as one of the most important techniques to investigate biofilms. Due to its non-destructive nature, biofilm morphology and physiology and the relationship between biofilm structure, adaptation, reactivity, and response to external stress can be investigated [27]. It is widely applied in characterizing the antimicrobial resistance mechanisms of biofilms [14], corrosion-inhibiting surface property analysis [28], and heavy metal tolerance [29]. CLSM has also contributed significantly to our understanding of the EPS matrix [9]. Structural heterogeneity of the biofilm matrix can be visualized and mapped by various fluorescent dyes that specifically bind to EPS compounds. The distribution of the EPS in aerobic acetate-fed and phenol-fed granules has been investigated by a quadruple staining scheme including four different dyes. Phenol stress significantly changes the distribution pattern of the different compounds in the EPS [30]. A direct response to solvent stress was described for the EPS of a *Pseudomonas* sp. biofilm. Upon the addition of styrene, the polysaccharide fraction of the EPS increased significantly [17]. Classical CLSM imaging is so far limited to approximately 250 μm thick biofilms in aqueous solutions, but penetration depths of up to 3 mm have been reported, when the aqueous phase has been exchanged for the amorphous fluoropolymer Nafion [31]. This is highly beneficial for a complete spatiotemporal visualization and mapping of the physiological status of the cells inside the deeper layers of the biofilms.

Atomic force microscopy (AFM)

AFM has been applied in elucidating corrosion phenomena and biofilm interaction, key events of biofilm developments such as surface conditioning, cell adhesion, and effects of antimicrobial compounds on biofilm development [32]. Recently, the biofilm colonization of metal surfaces by *Pseudomonas* NCIMB 2021 and *Desulfovibrio desulfuricans* was mapped by AFM and the detrimental pitting corrosion effect was quantitatively demonstrated [33]. AFM mainly provides information about the morphological details but little on the chemistry of the interface. Sometimes the soft biological samples may be damaged by the tip [33]. However, AFM is currently the only method that allows for investigation of the surface charge and the elasticity of the specimen. This is done by measuring the minute forces within or between biological molecules, something not possible by other techniques. This helps to understand the cell–surface interaction and the attachment phenomenon of bacteria [34].

Optical coherence tomography (OCT)

In an attempt to bridge the local and global structural properties of biofilms, OCT was introduced to study mesoscale (millimeter scale) 3D structures, thickness, and porosity of biofilms [35]. OCT should be combined with other techniques, for example CLSM or Raman microscopy, to fill the gap between micro- and macro-scale investigations. For example, such correlative and multi-microscopic approaches are applied to map river water biofilm composition [23]. CLSM, TEM, and scanning transmission X-ray microscopy (STXM) can be used in combination. Although the STXM and TEM are limited relative to CLSM in terms of sample preparation, fixation, and mechanical sectioning, their application in a correlative fashion was especially useful to gain insight into distribution of the polysaccharides, proteins, lipids, and nucleic acids in the EPS [23].

Microsensors

Microsensors play an important role in assessing solute concentration profiles in biofilms. As well as measuring various substances (such as O_2, H_2, H_2S, and others), temperature, pH and diffusivity can be measured [36,37]. Some electrochemical microsensors are commercially available. Most studies applying microsensors focus on oxygen as a key compound for biofilm growth. These studies revealed a steady decline in oxygen concentration from the surface of the biofilm into deeper layers. Biofilm structure strongly influences mass transport and it was found that 50% of the oxygen consumed by the biofilm was supplied by the voids [9]. Although the microelectrode tip may locally disturb the biofilm structure, temporal and spatial concentration gradient measurements have significantly contributed to the overall understanding of biofilms, especially regarding mass transfer. The introduction of non-invasive optodes enabled a combined and simultaneous microscopic imaging of biomass, important solutes such as O_2, and parameters such as pH [38]. Future research will benefit from the combination of confocal microscopy and microsensors for a better understanding of biofilm physiology, which is one of the key issues regarding biofilm productivity in respect of the status of the cells (e.g., VBNC state).

Cultivation setups for direct microscopic biofilm investigation

Biofilm investigation requires the development of cultivation systems with controlled flow and surface conditions to assess biofilm development, reactivity, and the resulting system response. Here, we focus on cultivation devices enabling a direct microscopic investigation of the biofilm. There are other cultivation devices not suited for direct investigation (e.g., rotating discs, modified Robbins devices, and Calgary devices), which are not discussed here [39]. Flowcells provide controlled hydrodynamic conditions and access to microscopic visualization of biofilms. They are relatively simple in design and compatible with both microscopy and microsensors, which allows a complete biofilm profiling [27,38,40]. Flowcells developed at the BioCentrum of the Technical University of Denmark have been used extensively to investigate antibiotic and shear stress [14]. A modified solvent resistant flowcell system was developed for solvent stress analysis [17]. Planar flowcell systems allow precise control of fluid flow conditions and user-defined environmental gradients. The influence of nutrient influx and hydrodynamic shear stress on *Pseudomonas aeruginosa* biofilm growth patterns was

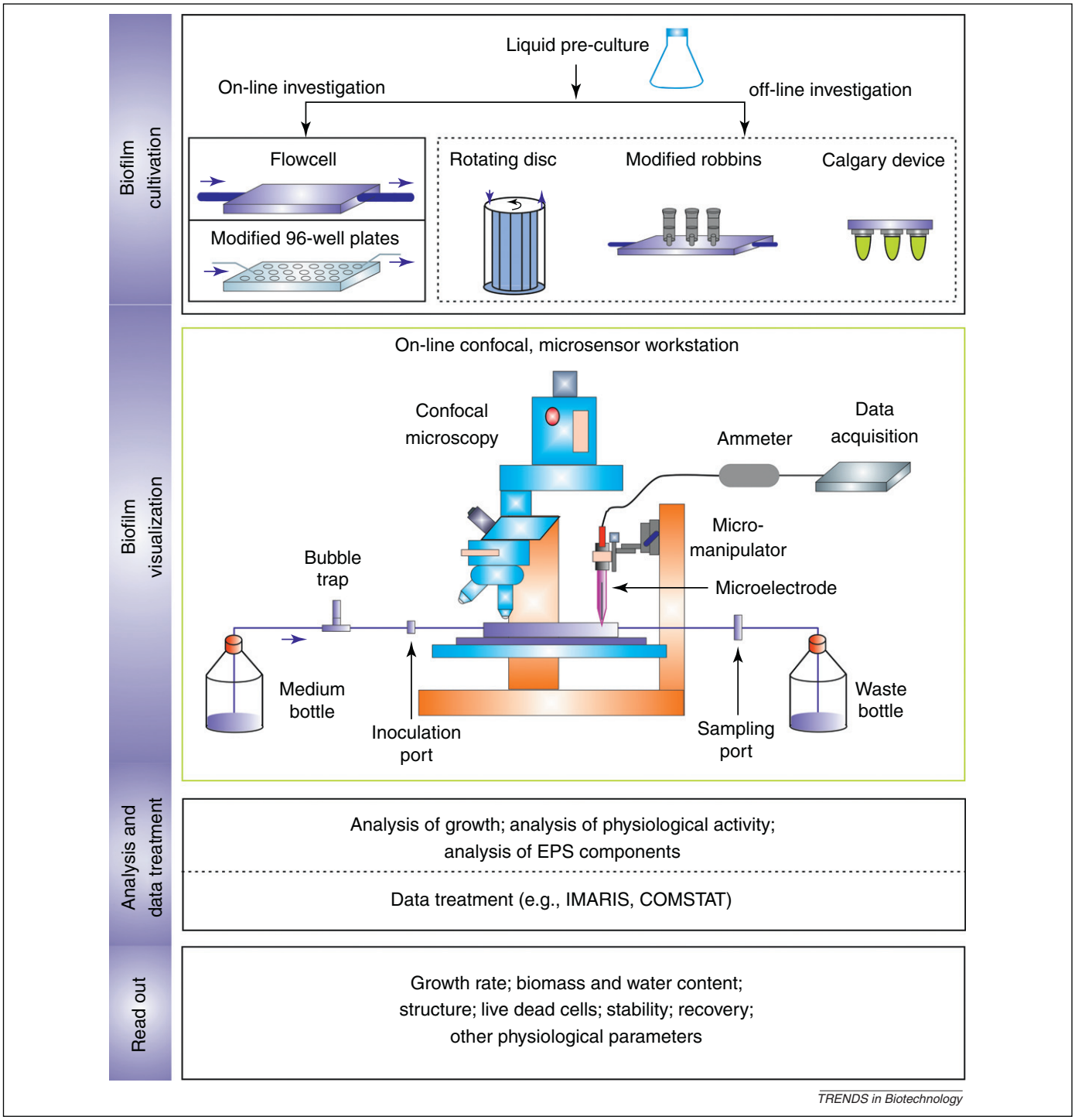

Figure 1. Toolbox for spatiotemporal catalytic biofilm characterization. This universal toolbox is based on simplified flow reactor systems, confocal microscopy, and microsensors. Detailed instructions on flowcell construction/operation are given in [14] and [17]. The microsensor integration with the flowcell is elucidated in [40] and [38]. Commercially available microelectrodes can be selected depending on requirements (e.g., oxygen and/or glucose). The use of *gfp*-expressing or otherwise fluorescent-labeled strains enables the microscopic visualization of the cells in the biofilm. Whereas the flowcell is mounted directly onto the microscopic workstation, other cultivation devices have to be interrupted (termed 'off-line') for the biofilm specimen to be monitored. Consequently, image analysis and micro-graphing with the microsensor can be performed. The resultant images from the microscopy are then transferred to software tools, for example IMARIS (Bitplane AG, Zürich, Switzerland) and COMSTAT [94] for quantitative analysis. The coupling of complementary techniques provides a detailed understanding of microbial ecology in reactor systems. If required, Raman microscopy or atomic force microscopy (AFM) can be integrated into the workstation.

studied with such systems [41]. In addition to the widely used flowcell systems, a modified 96-well microtiter plate with microfluidic channels allowing for continuous, on-line investigation of biofilm development was developed [42]. This system 'BioFlux' enables rapid, accurate, and high-throughput biofilm viability assays. With this background, we sketched out a universal toolbox for catalytic biofilm characterization (Figure 1).

Biofilms in biotechnological applications

Biofilms have long been used in fermentative processes for the production of bulk chemicals. Extensive reviews are

Box 2. Operational window for biofilm-based production processes

Bioprocesses heading for commercialization need to meet certain criteria. Among all, productivity, product concentration, and operation time are the most important parameters determining process feasibility. An operational window is defined as the operational space of a system (chemical, physical, and biological) and process constraints [71]. The operational space defines the minimum requirements and the maximum possible performance of a given reaction. Fitting in this case refers to the positioning of the individual process inside the defined operational space. This theoretical approach can be used to identify limiting parameters, and optimization strategies can be developed accordingly [71,72]. An operational window for biofilm-based production procedures is shown here, considering most of the reported biofilm-based bulk and fine chemical synthesis examples. Productivity and yield are the two most important parameters determining the economic feasibility of any biotransformation. In addition, final product concentrations (g l^{-1}) and total operation time (h) have to be taken into account [72]. The operational limits of the window are set on minimal and maximal product concentration, productivity, and operation time. A productivity of 0.1 g $l^{-1}h^{-1}$ ($P_{min,\ s}$) and a product concentration of 1 g l^{-1} were set to be the minimal requirement for fine chemical production operated in batch mode [72,73]. In general, biofilm-based bulk and fine chemical processes are expected to have high stability, which guarantees long running time. Hence, we assume 0.01 g l^{-1} h^{-1} ($P_{min,\ b}$) as the minimum productivity requirement for biofilm-based continuous operations. Kunduru and Pometto [44] reported the highest productivity (536 g l^{-1} h^{-1}; P_{max}) for biofilm-based ethanol production, and Qureshi *et al.* [10] reported a maximum operation time of 4 months in a fluidized bed reactor for butanol production. From these values, 6 months (4320 hours) of operational time were set as the maximum achievable limit (T_{max}). A running time (T_{min}) of 24 hours was assumed as the minimal requirement for the biofilm-based systems, including time required for biofilm development. The resulting operational window is shown in Figure I. Most of the bulk chemical production procedures fit well into this window, whereas most examples of fine chemical biotransformations are still located below the critical boundary. This is due to various factors such as toxicity of substrate or product.

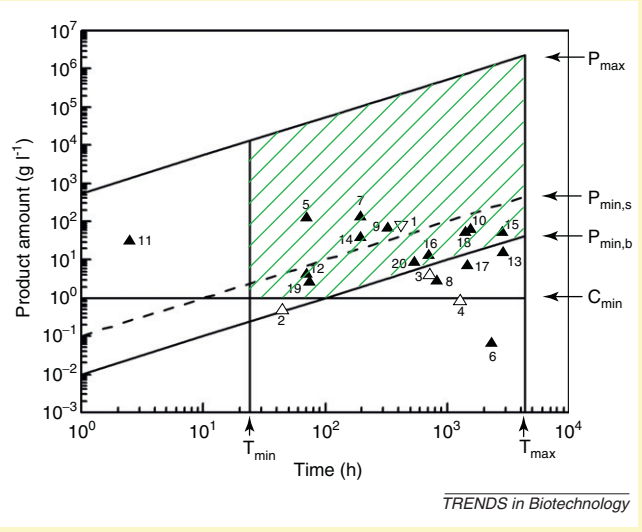

Figure I. Operational window for biofilm-based bulk and fine chemical production processes. $P_{min,\ b}$, minimum productivity limit for biofilm catalysts; $P_{min,\ s}$, minimum productivity limit for suspended cell catalysts; T_{max}, maximum process time; T_{min}, minimum process time; ▲, bulk chemical processes; Δ, fine chemical processes; ▽, fine chemical production by fermentative process. Numbers represent the processes given in Table 2.

available on reactor configurations and the type of chemicals produced [4,10,43]. Packed bed, fluidized bed, and membrane-aerated biofilm reactors are examples of some established reactor configurations. Volumetric productivities (g l_{aq}^{-1} h^{-1}) as high as 100-fold could be achieved with packed bed biofilm reactors for continuous ethanol fermentation, compared to suspended cells [44]. In addition to bulk chemicals, biofilms are evaluated for producing enzymes [45], fine chemicals [19–21], biofuels [46], and electricity in microbial fuel cells [12]. Although a lot of examples have been elucidated at lab or pilot scale, vinegar production by acetic acid bacteria in a 60-m^3 trickle bed biofilm reactor is the only commercial process reported at industrial scale to date [4,47]. To identify possible bottlenecks and the key parameters hindering biofilm process scale-up, a quantitative operational window is defined to evaluate these reported examples for their industrial relevance on a common platform (Box 2 and Table 2).

Biofilm reactors

A general feature of biofilm reactors is a continuous operation due to self-immobilized biocatalysts on inert solid supports known as substratum, which may be particles or membrane surfaces. In most cases, biofilm reactors offer high volumetric productivities (Table 2). The residence time of the cells is independent of the fluid phase flow rate and the biomass is not subject to washout [10]. Biofilms can be applied in various reactors. In this review, biofilm reactors are categorized as (i) well established and (ii) newly developed custom-made biofilm reactors. There have been several extensive reviews on the basic biofilm reactor types (packed bed reactors including trickle and submerged bed reactors, fluidized bed reactors, air lift reactors, up-flow anaerobic sludge blanket reactors, and expanded granular sludge bed reactors, as well as membrane-aerated biofilm reactors) [4,10,48]. These reactors have been widely applied in wastewater and off-gas treatment. Figure 2 shows operational strategies for newly developed custom-made biofilm reactors.

Recent advances in biofilm reactor engineering

Our understanding of biofilm biology is steadily increasing and various custom-made reactors for different applications are described. Based on the experience with well-established biofilm settings, a variety of newly developed custom-made biofilm reactors emerged for specific applications especially for fine chemical and enzyme production, biofuels, and other applications (Figure 2). These biofilm reactors are unique in their design and focus largely on often encountered bottlenecks in fine chemical syntheses such as (i) oxygen mass transfer, (ii) substrate and/or product solubility, toxicity, and diffusion, and finally (iii) efficient product recovery.

Membrane reactors or extractive membrane reactors have been widely applied in wastewater treatment [48]. Their application has been extended to complex biotransformations involving toxic, volatile, and poorly water-soluble substrates and products in combination with a two-phase system, where they are operated as membrane (silicone polymer-based)-aerated/attached biofilm reactors (MABR, Figure 2). The biofilm catalyst is always grown in the aqueous phase either on the inner membrane or on the

Table 2. Biofilm-based bulk and fine chemical production processes considered for the operational window

	Product	Reactor configuration	Substratum	Organism	Reactor volume (l)	Product concentration ($g\ l^{-1}$)	Maximum productivity ($g\ l^{-1}\ d^{-1}$)	Process time (h)	Productivity increment[b]	Refs
	Fine chemicals									
1[e]	Dihydroxyacetone[a]	Packed bed bubble column	Silicone coated Ralu rings	*Gluconobacter oxydans*	1.2	82	88.8	432	1.32	[7]
2	Benzylalcohol	Packed bed	Glass beads	*Zymomonas mobilis*	0.02	0.44	21	45	2	[21]
3	(*S*)-Styrene oxide	Solid support membrane[c]	Ceramic membrane	*Pseudomonas* sp. strain VLB120ΔC	0.01	3.94	28	744	n.i	[20]
4		Tubular membrane[c]	Silicone tube		0.0015	0.78	70	1320	n.i	[19]
	Bulk chemicals									
5	Acetic acid	Trickle bed	Beechwood shavings	Acetic acid bacteria	60 000	120	1.7	72	n.a	[47]
6	Hydrogen	Trickle bed	Polyurethane foam	*Caldicellulosiruptor saccharolyticus*	400	0.13	1.05	2232	n.a	[80]
7	Lactic acid	Rotating fibrous bed	Fibrous matrix	*Rhizopus oryzae*	5.0	126	60	200	1	[81]
8	Acetone + butanol + ethanol	Packed bed	Bonechar	*Clostridium acetobutylicum*	1.0	6.5	156	1500	65	[82]
9	Fumaric acid	Rotary biofilm contactor	Polysulfone plastic discs	*Rhizopus oryzae*	0.9	65	102	336	5	[83]
10	Lactic acid	Packed bed	PCS[d]	*Lactobacillus casei*	0.6	60	102	1584	2	[84]
11	Ethanol	Expanded bed	Vermiculite	*Zymomonas mobilis*	0.5	29.17	2520	2.5	26	[85]
12	Butanol	Packed bed	Brick pieces	*Clostridium Beijerinckii* BA101	0.312	4.04	48.24	72	5	[86]
13	Propionic acid	Packed bed	Cotton towel	*Propionibacterium acidipropionici*	0.29	15	37	2976	4	[87]
14	Ethanol	Packed bed	Ion exchange resins, ceramic chips	*Zymomonas mobilis*	0.16	37.77	3259	200	34	[88]
15	Ethanol	Fluidized bed	Macro-porous glass beads		0.11	50	312	2880	3	[89]
16	Acetone + butanol	Packed bed	Coke	*Clostridium acetobutylicum*	0.1	12	26.88	720	2	[90]
17	Butanol	Packed bed	Bonechar	*Clostridium acetobutylicum*	0.05	2.7	98.4	840	12	[91]
18	Ethanol	Packed bed	PCS	*Zymomonas mobilis*	0.03	50	2304	1440	19	[44]
19	Poly (3-hydroxybutyrate)	Packed bed	DEAE Sephadex A-25 beads	*Alcaligenes eutrophus*	0.03	2.5	0.80	75	n.i	[92]
20	Acetone + butanol + ethanol	Packed bed	Brick pieces	*Clostridium Beijerinckii* BA101	0.02	7.9	379.2	550	42	[93]

[a]Fermentative approach.

[b]Productivity increment is the productivity ratio of biofilms and comparable suspended cell processes.

[c]Productivity is calculated based on the aqueous phase volume of the reactor.

[d]Abbreviations: n.a., not available; n.i., no increment; PCS, plastic composite supports.

[e]The numbers in this column refer to Figure I in Box 2.

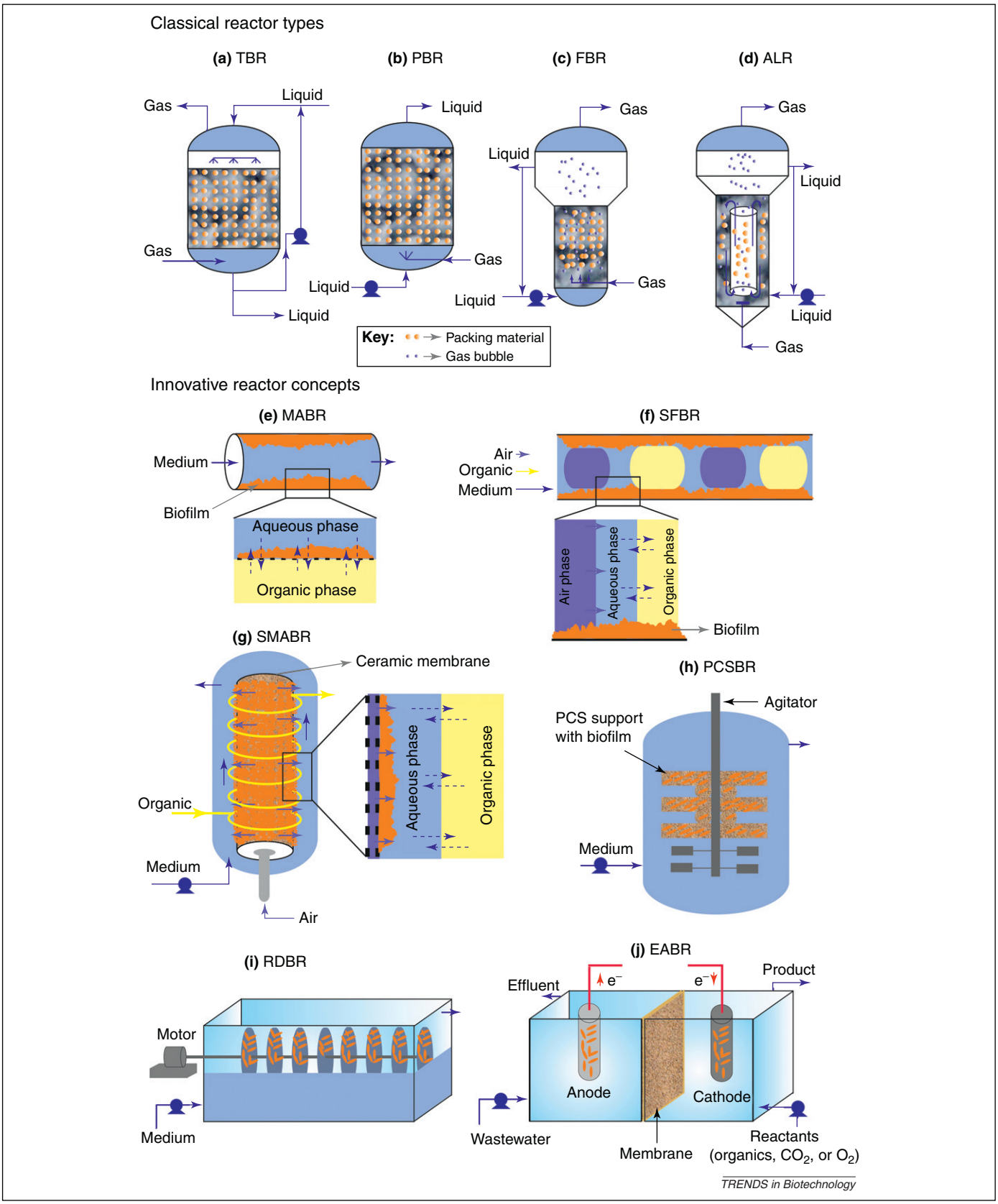

Figure 2. Biofilm reactor developments. **(a)** TBR, trickle bed reactor; **(b)** PBR, packed bed reactor; **(c)** FBR, fluidized bed reactor; **(d)** ALR, air lift reactor; **(e)** MABR, membrane aerated biofilm reactor; **(f)** SFBR, slug flow biofilm reactor; **(g)** SMABR, solid support membrane-aerated biofilm reactor; **(h)** PCSBR, plastic composite support biofilm reactor; **(i)** RDBR, rotating disc biofilm reactor; **(j)** EABR, electro-active biofilm reactor.

outer membrane surface depending on the location of the aqueous phase. The organic substrate is delivered through the membrane by diffusion and the product is continuously extracted out of the aqueous phase through the membrane into the organic phase [19]. Substrate/product solubility and toxicity can be overcome using this reactor configuration. However, the system suffers from insufficient oxygen supply and is not feasible for scale-up. Further development of this setup was the solid support membrane-aerated biofilm reactor (SMABR, Figure 2) growing biofilm directly on the aeration device and thus overcoming the oxygen limitation [20]. A microporous ceramic membrane uniformly supplied oxygen and served as substratum for biofilm growth. A silicone-based polymer membrane was used as a reservoir for toxic substrate and product. This type of reactor system offers scalable reactor configurations and a high oxygen transfer rate [20].

Microreactor configurations are attractive in terms of high mass and heat transfer rates because of large surface-to-volume ratios. This was exploited in a three-phase (liquid–liquid–gas) segmented flow system and it was possible to establish a stable *Pseudomonas* sp. biofilm. The segmented flow biofilm reactor (SFBR, Figure 2) complemented both MABR and SMABR, enabling direct oxygen transfer and avoiding the mass transfer barrier across a polymer membrane for substrate delivery. Shear forces prevented the system from being blocked by excessive biofilm growth [49].

Another approach utilizes plastic composite supports (PCS) as substratum for biofilm establishment containing tailor-made nutrients for biofilm growth, which are slowly released during cultivation. The PCS are attached to the reactor shaft (plastic composite support biofilm reactor, PCSBR; Figure 2). This unique combination resulted in a biofilm reactor for the production of lactic acid [50], bacterial cellulose, and the homo-polysaccharide Pullulan [51]. This reactor setup shortened the product formation lag phase due to the high cell density on the PCS supports. Excess biofilm growth was controlled via the agitation speed [50].

In an attempt to mimic the natural environmental growth conditions for the respective organism, different biofilm reactor configurations such as simple air membrane surface bioreactor and rotating disc biofilm reactor (RDBR, Figure 2) were developed [52,53]. Certain antimicrobial compounds are produced only when the respective organism grows as a biofilm, as shown for *Bacillus licheniformis* strain EI-34-6 producing the antimicrobial compound 'Bacitracin'. It was assumed that it functions as a kind of biofilm-specific inducer [52]. Another example of a strain producing antimicrobial compounds is *Streptomyces* sp. MS1/7. This estuarine salt-tolerant strain was grown as a biofilm on partially submerged rotating discs and high product titers (expressed as peak antimicrobial activity of 41 mm) were achieved [53].

Microbial-assisted electrosynthesis has recently gained increasing interest [54]. In an electro-active biofilm reactor (EABR, Figure 2), microbes grow on an anode and/or cathode and oxidize and/or reduce substrates to the required products such as biohydrogen [55], hydrogen peroxide, caustic soda [56], methane [54,57], ethanol [58], and other multi-carbon organic compounds [59]. Microbial electrosynthesis has the potential to become a key process in future bioproduction. Already today the production of hydrogen by biofilms is fourfold more efficient compared to classical systems based on water electrolysis [12]. However, implementation of such EBAR systems is so far not straightforward owing to various technological and economic reasons. Understanding the interaction between the organisms and the electrode surface, as well as the development of scalable scaffolds with suitable surface properties for biofilm attachment are the future key challenges of this interesting research field [60,61]. Electro-active biofilms in microbial fuel cells (MFC) oxidize organic substrates from wastewater or other resources as energy and carbon supply and produce electricity by transferring electrons finally to a cathode. An amazing amount of 1 kW m^{-3} (reactor volume) and 6.9 W m^{-2} (anode surface area) under ambient conditions can already be obtained. This is sufficient to satisfy the energy demand of a typical wastewater treatment plant [60]. The reported key challenges are a better understanding of the bacterial electron transfer to the electrode surface, the cathode performance, cost-effective materials and design, and finally the scale-up. Energy recovery during wastewater treatment and remote power generation are the two major areas where electro-active biofilms might become a stand-alone technology [60,62].

Another emerging topic is biofuel production from syngas by microbial cells. It is a promising alternative to conventional processes because microbial cells provide the advantage of whole biomass utilization thus complex pre-treatment steps and expensive enzymes are eliminated. However, poor mass transfer properties of syngas resulted in low ethanol yields [63]. MABRs proved to overcome the reported challenges. In a series of recent patent applications, different polymer membranes (PVDF, PVC, PES, etc., preferentially with a pore size less than 0.5 μm) have been used for the biotransformation of syngas catalyzed by biofilms [46,64]. In hollow fiber bundles, biofilm growth is achieved on the gas side rather than on the liquid side, which is different from the previous concept [65] where the biofilm grew on the liquid side leading to disadvantages such as excess biofilm growth and mass transfer limitations of the substrate. In the new setup, gas contact occurs on the side of biofilm localization and the liquid product diffuses through the membrane pores into the liquid stream. The direct contact of biofilm-grown cells with the gas phase and the stability of the syngas H_2:CO ratio significantly enhanced the reactor performance and ethanol production. This process is planned for commercialization by Coskata [63,66] and biofilms already play an important role in the race for efficient biofuel production.

Challenges and future targets

Biofilm formation is a multi-parameter phenomenon associated with the respective species, substratum, and environmental conditions. Every process design for the production of a given compound starts with the choice of an ideal biofilm catalyst and is followed by the development of a platform reactor and strategies for product recovery. Finally, the overall process control needs to be

well defined and optimized. The potential of biofilms as living biocatalysts has not been fully explored. This is due to the fact that biofilms react very specifically and are highly sensitive to their environment. A series of thoroughly characterized biofilm forming strains, with a toolbox for biocatalyst engineering, would facilitate the development of tailor-made catalysts [4]. Although the genetic stability, especially of plasmid-carrying recombinant strains, is regarded to be problematic in long-term applications, studies [67] using recombinant *Acetobacter xylinum* biofilms carrying a plasmid-encoded D-amino acid oxidase are encouraging. Recombinant *Escherichia coli* PHL644 encoding a tryptophan synthase has been reported as promising in this respect [68].

Multi-species biofilm applications are still in their infancy. Utilizing this concept for catalysis necessitates control of species composition. A simple and reproducible way to rationally construct a multi-species bacterial community uses sequential recirculation of *E. coli* cells with either *Pseudomonas aeruginosa* or *Bacillus subtilis* cells in a flowcell system called 'dial-a-biofilm'. This approach may be useful to perform cascade or multi-step reactions in drug development or fuel synthesis [69].

Can we apply the general methods such as fundamental, semi-fundamental, rule of thumb, and dimensionless numbers for biofilm reactor scale-up? Because biofilm reactor performance is influenced by parameters such as oxygen transfer, active biofilm thickness, and substrate mass transfer, a thorough understanding of these process parameters is necessary. Scaling-up bioprocesses while keeping all conditions in the optimal range is usually not possible and thus it is necessary to set priorities. Scale-up of aerobic biofilm processes may be performed on the basis of the volumetric oxygen mass transfer coefficient ($k_L{\cdot}a$). For MABRs, numbering-up or parallelization would be an option, provided that sufficient oxygen mass transfer to the system is ensured. Despite many successful lab-scale examples, no transfer at the commercial scale has been reported yet, except for acetic acid production. More effort in bioengineering as well as biotechnology is required to develop feasible scale-up strategies and biological process control (Box 3).

Box 3. Outstanding questions

- What possibilities do we have for controlling biofilm formation and growth? As discussed in [22], '...once individual biofilm regulatory circuits are understood, biofilm formation and dispersal can be manipulated'. However, although promising findings have been reported in the area of growth control via natural molecular mechanisms, there is still some ground to cover before we will be able to engineer biofilm growth on a global level. In addition, biofilm growth may also be controlled by technical means, as high shear stress in capillary systems.
- Do multi-species biofilms have a future in bioprocess development? The establishment of multiple species catalyzing a series of reactions in a type of reaction cascade seems to be a very interesting field of research. However, to achieve this goal, much more fundamental research is necessary to understand the interspecies dependencies on which these microbial communities are based.
- Are we equipped with innovative concepts needed for product recovery? One of the rarely addressed downsides of biofilm-based processes is the highly diluted bleed stream, which results in problems in product work-up. This is an important issue for water-soluble compounds because these cannot simply be extracted by an organic solvent as shown in various examples [19,20]. Therefore, future biofilm process development needs to focus on an integrated product isolation technique to facilitate downstream processing.
- How can we shorten development times for biofilm catalysts? One of the key problems of bioprocess development is the time necessary to design a suitable bioprocess for a given reaction. This is even more pronounced for biofilms because biofilm-specific characteristics may be very diverse among different strains and it is difficult to define general rules. Future research should focus on the development of a series of workhorses for different chemical syntheses in platform biofilm reactors, as proposed by [4].

Concluding remarks

Catalytic biofilms are highly heterogeneous and dynamic in space and time, which makes their analysis and control rather challenging. Their growth and tolerance behavior cannot be revealed simply by a single stand-alone technique. The biggest challenge in the near future will be to integrate the respective analytical tools directly into the bioreactor to obtain reliable 'real-process' data and engineer biofilms using the findings of fundamental research to tailor them according to the respective process needs. Future research should enable the development of a series of workhorses for different chemical syntheses in platform biofilm reactors. If growth rates can be controlled, such reactors can run for months, considerably reducing reactor downtimes. Various research activities in recent years showed the huge potential of biofilms for diverse biotechnological applications. However, a strong commitment of industry to implement such a new technology is crucial for driving this approach to a successful outcome.

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