A rotating bioreactor for the production of biofilms at the solid–air interface

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Abstract
Conventional bioreactors are typically developed for the production of planktonic bacteria or submerged biofilms. In contrast, reactors for the continuous production of biofilms at the solid–air interface are scarce, and they require specific conditions since the bacteria need to attach firmly to the surface and require a permanent supply of moisture and nutrients from below. Recently, research from the field of civil engineering has pinpointed an increased need for the production of terrestrial biofilms: several variants of Bacillus subtilis biofilms have been shown to be useful additives to mortar that increase the water repellency, and, thus, the lifetime of the cementitious material. The bioreactor introduced here allows for the continuous production of such bacterial biofilms at the solid-air interface, and they have virtually identical properties as biofilms cultivated via classical microbiological techniques. This is made possible by equipping a rotating cylinder with a porous membrane that acts as a solid growth substrate the bacterial biomass can form on. In this configuration, nutrient supply is enabled via diffusive transport of a suitable growth medium from the core volume of the cylindrical reactor to the membrane surface. In addition to cultivating bacterial biofilms, the versatile and adaptable setup introduced here also enables the growth of other microbial organisms including the yeast Saccharomyces cerevisiae and the fungus Penicillium chrysogenum.

KEYWORDS
bacteria, biomass production, bioreactor, fungi, hydrophobic mortar

1 | INTRODUCTION

According to Yin and Yang philosophy, there is something “bad” in every “good” and vice versa. Similarly, bacterial biofilms, which are typically considered to be highly undesirable contaminants in industrial or medical setups, can have their perks as well. Indeed, for many biotechnological applications, this persistent form of bacterial communities is actually preferred over planktonic cells (Keskin et al., 2012; Morikawa, 2006; Qureshi et al., 2005)—and this can be attributed to the same astonishing material properties that render biofilms annoying when found on surfaces such as pipes or implants: they can be sticky (Kretschmer et al., 2021; Yan et al., 2018), sturdy and resilient (Lewis, 2005; Yan & Bassler, 2019), and liquid-repellent (Epstein et al., 2011; García et al., 2019). Recent research efforts have...
identified a broad range of beneficial applications where bacterial biofilms can serve as interesting components, and example areas include agriculture, biomedicine, (environmental) biotechnology, electricity generation, and construction engineering (Hayta et al., 2021).

The latter application area might seem the most surprising—even though biological additives to construction materials were already explored by the ancient Egyptians and Romans and during the Qing dynasty (Dai et al., 2019; Stark & Wicht, 1998). Recently, the idea of using bacterial biofilms as additives to cementitious materials has been rediscovered, and the resulting hybrid mortar has been shown to possess highly interesting water-repellent properties (Ertelt et al., 2020; Grumbein et al., 2016). Of course, for such a bio-enhanced material to be used in real construction engineering efforts, larger biofilm amounts are required than can be grown in the lab using classical microbiological assays. However, there are only a few examples of dedicated bioreactors that produce bacterial biofilms—and in those kinds of bioreactors, the bacteria are either attached to (suspended or packed) support materials or directly to the bioreactor surface. Examples of biofilm bioreactors using support materials include sequencing batch reactors (Arrojo et al., 2004; Irvine & Ketchum, 1989; Münch et al., 1996), continuous stirred tank reactors (Kunduru & Pometto, 1996; Ramasamy & Abbasi, 2000; Tyagi & Ghose, 1982; Xu, 2019), packed- or fluidized-bed reactors (Mallikarjuna & Dash, 2020; Özkıaya et al., 2019; Sahu et al., 2009; Shen et al., 2017; Shieh & Keenan, 1986), and trickling bed reactors (Devarapalli et al., 2016; Jensen et al., 2021; Tsapekos et al., 2021).

Rotating disc contractors or membrane bioreactors are common examples where biofilm production occurs on the bioreactor surface, and those are typically used in wastewater treatment plants (Costa et al., 2017; Melo, 2003; Renaudie et al., 2021; Taşkan et al., 2019). The latter bioreactor variant also comes with the advantage of easily separating the biofilm after usage and enhancing biofilm attachment and growth by chemically modifying the reactor surface (Lackner et al., 2009; Terada et al., 2004; Yamamoto et al., 1989).

In most of these bioreactors, the biofilms perform a dedicated function in situ: The bacteria attach to organic or inorganic support materials, generate a biofilm, then catalyze reactions within the bioreactor, and finally are removed or inactivated once their task is fulfilled (Muffler et al., 2014). For an application in construction engineering, however, the biofilm itself is the desired product that needs to be harvested from the bioreactor. Thus, for the bioreactor to be sustainable, the support material used for biofilm cultivation needs to be reusable.

Here, we introduce a novel type of bioreactor that allows for cultivating bacterial biofilms on a solid substrate. This reactor makes use of a cylindrical geometry such that the substrate inoculation with bacteria and the biofilm harvesting step are conducted at different locations of the cylinder surface. Nutrient supply is guaranteed via a combination of diffusion and convection effects that drive a nutrient solution from the core of the cylinder to its outer surface. Here, a combined agar/membrane layer supports the growth of different bacterial biofilms as well as yeast and fungus biofilms. As a consequence of this particular design, when selecting a sufficiently low rotation speed, a continuous biofilm growth/harvest cycle is enabled.

We show that Bacillus subtilis natto biofilms harvested from this bioreactor have virtually identical material properties and hydrophobicity-conveying abilities to mortar as when they are grown in a classical microbiological manner, that is, on agar-filled Petri dishes.

2 | MATERIALS AND METHODS

2.1 | Microorganisms and growth conditions

Two different bacterial strains were used in this study: B. subtilis natto (27E3), obtained from the Bacillus Gene Stock Center and B. subtilis 3610, obtained from the lab of Roberto Kolter (Harvard Medical School). Additionally, the yeast Saccharomyces cerevisiae (Dr. Oetker) and the fungus Penicillium chrysogenum DSM 848 (DSMZ) were used. For the cultivation of bacterial biofilm on agar plates and membranes, liquid cultures of B. subtilis natto and B. subtilis 3610 were generated by incubating small pieces of a frozen glycerol stock in 10 ml of 2.5% (wt/vol) lysogeny broth (LB) medium (Luria/Miller; Carl Roth GmbH) at 37°C while shaking at 300 rpm for ≈18 h. For biofilm growth experiments conducted on the bioreactor, the same liquid cultures were prepared in 500 ml of 2.5% (wt/vol) LB medium at 37°C while shaking at 100 rpm for ≈18 h.

Yeast liquid cultures were prepared by inoculating a small piece of dried yeast into 10 ml of yeast extract peptone dextrose (YPD) medium, which contains 1% yeast extract (Carl Roth GmbH), 2% peptone (Carl Roth GmbH), and 2% glucose (Sigma-Aldrich Corp.). The culture was incubated at 30°C while shaking at 300 rpm for ≈18 h.

To cultivate fungus, a potato/dextrose medium was prepared. In brief, 200 g potato was peeled and sliced into small cubes. After boiling those pieces in 1 L distilled water for 1 h, the infusion cleared by sieving, 20 g glucose was added to the infusion and the volume was adjusted to 1 L. Then, a piece of frozen glycerol stock of P. chrysogenum was inoculated in 10 ml of this potato dextrose medium, and the culture was incubated at 24°C while shaking at 300 rpm for 3 days.

2.2 | Microbial cultivation on membranes and agar

To find an optimal substrate for the growth of different microorganisms (bacteria, yeast, and fungus), several commercial membranes with a pore size of 0.2 µm each were tested: cellulose nitrate (obtained from analytical funnels for microbiology, Sigma-Aldrich Corp.), surfactant-free cellulose acetate (SFCA; Nalgene™ Rapid-Flow™ Membrane; Thermo Fisher Scientific), hydrophilic polyvinylidene difluoride (hydrophilic PVDF; Microlab Scientific Co., Ltd.), hydrophobic PVDF (Immobilon-PSQ Membrane; Merck KGaA), and polycarbonate (PC; ipPore™ Track Etched Membrane; it4ip S.A.) membranes. Additionally, to hydrophilize the commercial hydrophobic PVDF membranes, these membranes were immersed into isopropyl alcohol (i-ProOH, HPLC grade > 99.9%; Sigma-Aldrich Corp.) for 12 h. Afterward, the samples were immersed into deionized water for 6 h to replace most of the alcohol with water. The success of this hydrophilization procedure was verified by determining the contact angle formed by a small water droplet (4.5 µl) on the membranes using the
video-based optical contact angle measuring system Drop Shape Analyzer (Krüss-Easy Drop) and the software ADVANCE-sessile drop. Those measurements were repeated five times on each membrane.

For bacterial growth tests, round samples with a diameter of 3 cm were cut out from each membrane variant and sterilized by exposure to UV-light for at least 15 min. Then, these sterile membranes were placed onto 1.5% (wt/vol) agar gel patches of the same diameter having a thickness of ∼2.4 mm. The membrane-covered agar patches were inserted into tailored polytetrafluoroethylene holders with large holes, which then were placed into the wells of a six-well plate. Each well was filled with ∼8 ml of LB medium, YEPD medium, and potato/dextrose medium, respectively. Afterward, the amount of added liquid media was adjusted until direct contact with the agar patches was achieved. Then, 13 µl of an overnight culture (diluted to an optical density [OD] of 0.7) of either bacteria (B. subtilis natto and 3610) or yeast (S. cerevisiae) were distributed across these membrane pieces. To grow P. chrysogenum biofilms on the membranes, the same procedure was followed yet without diluting the fungal overnight culture. The six-well plate was then covered and a sterile towel was placed between the samples and the cover to prevent condensate water from dropping onto the growing biofilms. To promote the diffusion of nutrients from the medium reservoir towards the upper membrane surface, a temperature gradient was created by incubating the inoculated six-well plates on a heating plate at 37°C for 2 days (with the top of the plates exposed to room temperature). Owing to the different conditions required for fungus growth, the fungus biofilms were grown at room temperature (without using a heating plate) for 4 days. For all samples, the liquid medium was refreshed daily. After the respective incubation time, the formed biofilms were harvested from the membrane surfaces by collecting them with a spatula, and their mass was determined by weighing (Balance XSE205 DualRange; Mettler Toledo GmbH).

As control samples that allow for assessing putative diffusion-hindering effects of the different membrane variants, all microorganisms were also inoculated directly onto agar patches and incubated at the same conditions as described above. The second set of control samples was created to quantify putative growth retardation effects arising from the diffusion-limited nutrient supply present in those membrane-covered agar patches (which resembles the final condition on the bioreactor). For this purpose, standard agar plates with a diameter of 90 mm were prepared and enriched with 2.5% LB medium; thus, in those samples, the nutrients were readily available at the surface of the agar layer at the start of microbial inoculation. Then, 100 µl of B. subtilis natto culture (diluted to an OD of 0.7) were distributed on these LB-enriched agar plates and incubated in an incubator at 37°C at high relative humidity (80%-90%) for 2 days. This high relative humidity was achieved by a water bath that was placed into the incubator. Similarly, in the bioreactor tests, evaporation of liquid from the bacterial bath contributed to high humidity in the incubator. This bacterial bath, in turn, was continuously replenished with liquid medium from the core of the reactor, as it slowly dripped into the bacterial bath at the lower apex of the cylinder thus maintaining the fluid level in the bacterial inoculation bath.

For each microorganism/substrate combination, three independent samples were prepared from each growth batch, and all experiments were repeated three times using a different growth batch each time.

2.3 | Bacterial cultivation on the bioreactor

To grow B. subtilis natto biofilm on the bioreactor, the bioreactor was fully assembled and an agar layer was applied to the outer surface of the cylinder. To do so, the cylinder was manually rotated while partially immersed into a bath of liquid agar (1.5% [wt/vol] agar–agar; Carl Roth GmbH) heated to a temperature of ≈60°C; a full rotation was conducted, then the cylinder was removed from the agar bath until the applied agar layer had solidified. This procedure was repeated three times, after which an agar layer of ≈3–5 mm thickness was obtained. Additionally, 2 ml liquid agar was inserted into each hole of the cylinder (from its inside) to create an additional resistance that helped prevent the liquid medium filled into the core volume of the cylinder from leaking out.

A hydrophilic PVDF membrane (Microlab Scientific Co., Ltd.) was then added on top of the agar layer and fixed as follows: A 10 mM solution of 1% DOPA (dopamine hydrochloride; Sigma-Aldrich Corp.; dissolved in pH 8.5 Tris buffer; Illinois Tool Works Inc.) was applied in circular lines onto the agar surface to glue the membrane to the agar layer; in addition, a DOPA–hyaluronic acid (HA) conjugate was used to glue the membrane overlap regions to each other. For this DOPA–HA conjugate, a solution of 1% HA (HA sodium salt, Streptococcus equi, 91%; Thermo Fisher Scientific), 0.1% 1-ethyl-3-(3-dimethylaminopropyl) (EDC) carbodiimide–hydrochloride; 99%; Carl Roth GmbH), and 0.1% N-hydroxysuccinimide ([NHS], 98%; Sigma-Aldrich Corp.) was prepared in 10 mM MES and buffered to pH 5. The HA solution and EDC/NHS solution were dissolved separately. Additionally, a solution of 1% DOPA (dopamine hydrochloride; Sigma-Aldrich Corp.) was prepared in phosphate-buffered saline buffer at pH 8. After stirring overnight, the solutions were mixed and dialyzed for 72 h at 4°C using a dialysis membrane (Spectra/Por®7 Dialysis membrane pretreated regenerated cellulose tubing, MWCO: 50 kD; Spectrum Chemical Mfg. Corp.). The dialyzed solution was frozen at −80°C and lyophilized (Alpha 1-2 LDplus; Martin Christ Gefriertrocknungsanlagen GmbH). In a final step, the freeze-dried DOPA conjugate was dissolved at 8 mg mL−1 in ddH2O, poured in a Petri dish, and dried at 60°C to generate a thin adhesive film.

To supply the bacteria on the outer membrane surface with nutrients, the inside of the cylinder was infused with 5% (wt/vol) LB medium (Luria/Miller; Carl Roth GmbH). For the first 24 h, the reactor was run without bacteria to ensure that the liquid medium has enough time to diffuse from the inside to the outer surface of the cylinder. During the first 24 h, in which no bacteria were applied to the membrane surface, the cylinder surface was covered with a UV-sterilized plastic foil (plastic wrap; Zentrale Handelsgesellschaft GmbH) to protect the surface from contaminations from the atmosphere. For UV sterilization of this foil, both sides of the foil were exposed to UV light for at least 30 min.
After 24 h, the cylinder was partially immersed (2–3 cm) into a bacterial bath, that is, a liquid culture of planktonic \textit{B. subtilis} natto bacteria having an optical density of 0.1–0.15. After 24 h, the bacterial bath was renewed and the polydimethylsiloxane (PDMS) blade was installed to initiate the biofilm harvesting process.

To examine the microbial composition of the cultivated biofilm (i.e., to test for contaminations with other bacteria), we collected biofilm samples from three different areas of the cylinder and determined their mass by weighing. Then, these biofilm samples were added to 2 ml of a physiological saline solution (0.9% wt/vol NaCl), and then rigorously stirred at \( \approx 2500 \) rpm for 20 min followed by a short vortexing step. The such obtained bacterial solution was serially diluted until a dilution of \( 10^{-6} \) was obtained. Then, 100 µl of each diluted solution was inoculated on LB agar plates in duplicates and distributed by adding glass beads and shaking the plates. The inoculated agar plates were then incubated at 37°C for 24 h. For each dilution step, the grown colonies were evaluated regarding their morphological appearance, and the number of viable bacteria within the initial biofilm (colony-forming units/mg biofilm) was calculated from the last two dilutions.

### 2.4 | Rheology

To compare the viscoelastic properties of biofilms cultivated under the different conditions described above (i.e., on agar-filled Petri dishes and on the membrane surface of the bioreactor), rheological measurements were conducted. For this purpose, a commercial shear rheometer (MCR 302; Anton Paar) equipped with a 25 mm steel measuring head (PP25) and a plate–plate geometry was employed. The plate separation was set to 0.3 mm, and a solvent trap was installed to prevent sample drying during the measurements that were realized at 21°C and in strain-controlled mode. To ensure a linear material response, small strains corresponding to a torque of \( \approx 0.5 \) µN·m (this corresponds to a shear stress of \( \approx 0.1 \) Pa) were applied. In every rheological experiment, both the storage and loss modulus were determined over a frequency range of 0.1–10 Hz. Since, in all cases described in this study, the moduli obtained for a given biofilm sample were only weakly dependent on frequency, the obtained storage moduli were averaged over the complete measured frequency spectrum to obtain the bar plots shown in the manuscript. Obvious outliers resulting from measuring artifacts were excluded from calculating these mean values. For each sample type, at least three samples were tested, which were obtained from different locations of the reactor surface and from different agar plates, respectively.

### 2.5 | Biofilm-enriched mortar samples

#### 2.5.1 | Sample preparation

To cultivate biofilm on agar plates, 100 µl of bacterial overnight culture were plated onto each agar plate (1.5% vol/wt, Agar–Agar, Kobe I; Carl Roth GmbH; enriched with LB medium [2.5 vol/wt]) and incubated at 37°C for 24 h. From those agar plates, the grown biofilm was harvested by manually scraping the plates. On the bioreactor, biofilm was grown as described above and manually collected from the PDMS blade. Both biofilm variants were freeze-dried for 3 days and afterward ground into a fine powder having an average particle size of \( \approx 500 \) µm. In full analogy to previous experiments with such freeze-dried biofilms (Grumbein et al., 2016), the biofilm concentration added to mortar is described by the parameter biofilm content, which describes the ratio of fresh biofilm with respect to the mass of dry cement used for mortar sample generation. Using the mass loss factor, which is defined as the ratio of fresh biofilm with respect to the mass of lyophilized biofilm, the required amount of lyophilized biofilm can be calculated to match the respective amount of fresh biofilm.

#### 2.5.2 | Water repellency tests

To evaluate the wettting resistance of different mortar samples, small mortar samples were prepared by mixing a 3:1 mixture of CEN standard sand (NORMENSAND GmbH) and cement (Portland cement CEM 42.5 N; Schwenck Zement KG) with a suspension of biofilm powder in the respective amount of water. All mortar samples were prepared with a w/c ratio of 0.5 and cured for 3 days at room temperature before contact angle measurements were conducted. For measuring contact angles, a drop shape analyzer (DAS25S; Krüss GmbH) was used. Five µl droplets of ddH₂O were placed onto each sample at different spots, and images were acquired from a lateral view using the built-in high-speed camera (CF04; Krüss GmbH). The contact angle was then evaluated using the software ADVANCE (Krüss GmbH).

For capillary water uptake experiments, samples were prepared according to DIN EN 196-1 using an automatic laboratory mortar mixer (ToniMix; Zwick Roell). Cement and biofilm powder was added to the desired amount of water within 10 s, and the mixing process was immediately started at a stirring speed of 140 rpm. After 30 s, sand was added within a time window of 30 s, and the mixture was stirred at a stirring speed of 285 rpm for an additional 30 s. Then, the mixing process was stopped for 90 s. During this break, mortar adherent to the stirring head and/or the upper part of the bowl was transferred back to the bottom of the bowl using a rubber scraper, and the stirring process was continued for an additional 60 s at a stirring speed of 285 rpm. The prepared mortar was then poured into the desired mold within 120 s, while being compacted using a vibrating table.

### 3 | RESULTS AND DISCUSSION

#### 3.1 | Bioreactor design

\textit{B. subtilis} biofilms form at the solid-air or liquid-air interface, and they require a moisture and nutrient supply from their growth substrate.
For instance, a thin biofilm layer – so-called pellicles – can be formed on the surface of standing liquids. Yet, here, the generated biomass is very low. The most common laboratory procedure to cultivate *B. subtilis* biofilm makes use of agar-filled Petri dishes, where the nutrients (typically LB medium) are added to the agar layer. However, due to the nutrient depletion from the agar substrate during biofilm growth, this procedure does not allow for continuous biofilm cultivation. Moreover, biofilm harvesting from such agar plates needs to be conducted manually and carefully to ensure that only biofilm is removed during the harvesting procedure while avoiding contaminations of the bacterial biomass with small agar pieces. Owing to those limitations, this traditional biofilm cultivation process is very material- and work-intensive and thus not suitable for the production of larger biofilm amounts.

To solve those limitations, we here introduce a new type of bioreactor that meets the requirements mentioned above but allows for a continuous growth/harvesting cycle of biofilm on/from the same substrate. The basic principle of this new bioreactor is depicted in Figure 1. It comprises a hollow cylinder that is rotated along its long axis. At its lower apex, the cylinder is immersed into a bacterial liquid culture which – during rotation – applies a bacterial coat to the cylinder surface. This cylinder has a length of 26.8 cm and an outer diameter of 19.9 cm, which results in an outer surface area of 1675 cm². It is suspended horizontally by a framework, which allows for adjusting the z-position of the cylinder (Figure 1a). As a material for this cylinder, we selected hydrophilic polypropylene (PP-H AlphaPlus; Simona AG). This material was chosen as it can be autoclaved (for sterilization) and offers mechanical properties suitable for machining. However, growing biofilms directly on this PP material is very difficult; thus, a membrane layer is added on top to facilitate bacterial attachment and growth (vide infra).

Nutrient supply to this membrane layer is enabled by a combination of two measures: first, cylindrical holes with a diameter of 1 cm are drilled into the PP cylinder; this allows a nutrient solution added to the inner volume of the cylinder to reach the outer surface. Resupply of this nutrient solution is guaranteed by connecting the inner volume of the cylinder to a tank via tubings. Second, an agar layer of 3-5 mm (1.5%; Carl Roth GmbH) is sandwiched between the cylinder and the membrane; during this step, the holes in the cylinder are filled with agar as well. This agar layer acts as a sponge-like structure that guarantees nutrient transport to the membrane surface via capillary forces while preventing uncontrolled leakage of the nutrient solution from the bottom part of the bioreactor. The membrane is fixated to this agar layer in two ways: at the edges of the cylinder, two rigid polyvinyl chloride rings clamp the membrane to the cylinder; in addition, thin lines of L-DOPA glue the membrane to the agar substrate and stabilize the membrane/membrane overlap region (Figure 1b).

The last structural component of the bioreactor is responsible for biofilm harvesting: We installed a PDMS blade, which is brought into mechanical contact with the membrane surface via elastic springs (spring rate: 0.7 N mm⁻¹; Figure 1a). Images of the fully assembled bioreactor are shown in Figure 2. With this configuration – once the bioreactor is set into the rotation – the PDMS blade can collect the cultivated biofilm from the membrane surface without damaging the membrane (Figures 1c and 2a). Such rotation is enabled by a 12 V direct current motor (BQLZR DC 12 V 0.6 RPM; Figure 2c), and the rotation speed is chosen to be slow enough that a continuous biofilm layer can form during 5/6 of a full rotation (i.e., a full rotation requires 12 h to conclude). To implement this slow speed, the rotational movement is clocked, so that the cylinder rotates for 60° and then pauses again. When the cylinder completes one full rotation within 12 h, the according time span of a single rotation phase amounts to 3 min and the time span of a single waiting step amounts to 117 min. Once the harvesting step is completed, the membrane surface is immersed again into the bacterial liquid culture, and the cycle starts over. The whole bioreactor is placed into an incubator (CB-S 170; Binder GmbH; Figure 2b), where the temperature (typically, 37°C) and the relative humidity (we here selected 80%–90%) can be controlled. The biofilm harvested from the reactor by the PDMS blade that is slightly pressed against the membranes by springs (Figure 2d,e)
can then manually be collected from this blade, for example, once every 24 h.

3.2 | Membrane selection for microorganism cultivation

To cultivate microorganisms on the cylinder, a membrane layer is used. Employing such a membrane surface is preferred over direct cultivation on the agar layer since such membranes are more durable than the brittle agar; thus, the membranes should be able to withstand multiple harvesting cycles without being damaged. To identify a membrane material that combines suitable material properties with good microbial growth behavior on its surface, we compare the performance of four different membranes, which were reported in the literature to allow for microbial cultivation (Bric et al., 1991; Mohammad et al., 2019): a cellulose nitrate membrane, an SFCA membrane, two PVDF membranes (hydrophilic and hydrophobic, respectively), and a PC membrane (Figure 3a) – and all those membrane variants were selected to have a pore size of 0.2 µm each to prevent inoculated bacteria from penetrating the membrane layer.

In a first step, the membranes are inoculated with *B. subtilis natto* bacteria and then incubated to allow for biofilm formation. For those growth tests, a custom-made setup is chosen (see Figure 3b) to mimic the growth conditions present on the surface of the bioreactor. In brief, with this setup, nutrient supply to the membrane surface is enabled by a combination of diffusion and convection effects (see Section 2 Materials and Methods for details). After an incubation time of 2 days, we find equal or even higher amounts of *B. subtilis natto* biofilm on almost all membrane variants than when bacterial inoculation is conducted directly on agar (Figure 3c). Only on hydrophobic PVDF membranes, biofilm formation is negligibly low. However, after treating those hydrophobic PVDF membranes with i-PrOH, the membranes become slightly hydrophilic as verified by an alteration of the contact angle from 149.31° ± 5.30° to 83.93° ± 9.01°; consistently, biofilm growth on these hydrophilized membranes is comparable with the growth on commercial hydrophilic PVDF membranes which obtain a contact angle of 34.65° ± 5.05°.

Importantly, “classical” cultivation of this biofilm on standard agar plates (where the agar layer has the same agar thickness as in the diffusion experiments but the nutrients are added to the agar layer during its production) returns less biomass than growth on the different membrane layers. We attribute this finding to a limitation of nutrients in those classical agar plates: here, as soon as all the accessible nutrients from the agar layer are consumed by the bacteria, biofilm growth will come to an end. In contrast, owing to the ongoing replacement of the growth media in the liquid reservoir used for the diffusion experiments with membrane layers (which imitates the continuous nutrient resupply that will be enabled on the rotating bioreactor), the nutrient limitation is not an issue there. However, also for biofilm cultivation of those membranes, a boost of the biomass output can be obtained if the nutrient concentration in the growth medium is increased (Figure 3d).
Experimental tests for membrane selection. (a) Photographic images of the different membrane materials before (upper row) and after (lower row) folding. (b) Experimental setup of the growth experiments, in which the microorganisms are cultivated on top of a membrane and supplied by nutrients from a liquid reservoir by diffusion through a porous agar patch. (c) Photographic images of different microorganisms cultivated on PVDF and PC membranes using the setup depicted in (b). (d) Harvested biomass of *Bacillus subtilis* (*B. subtilis*) *natto* biofilms; they were either cultivated on standard agar plates or with the growth setup depicted in (b); for the latter, different membrane materials and, for the hydrophilic PVDF membrane, two different nutrient concentrations (25 and 50 g L\(^{-1}\)) were investigated. With the same membrane-based setup, the amount of harvested biomass obtained on hydrophilic PVDF and PC membranes, respectively, is shown for *B. subtilis* 3610 biofilms (e), *Saccharomyces cerevisiae* (*S. cerevisiae*) cultivation (f), and *Penicillium chrysogenum* (*P. chrysogenum*) cultivation (g). #no biofilm growth; ##biofilm growth occurred, but harvesting was impossible without destroying the substrate. Scale bars in (a) and (c) correspond to 1 cm. Data shown in (d)–(g) represents the mean and the standard deviation as obtained from at least six independent samples from at least two growth batches. LB, lysogeny broth; PC, polycarbonate; PTFE, polytetrafluoroethylene; PVDF, polyvinylidene fluoride; SFCA, surfactant-free cellulose acetate.
When handling different membrane variants, it becomes apparent that the cellulose nitrate and SFCA membranes are not ideal for the use intended here: when they are folded, they break apart. This indicates that their lifetime on a rotating bioreactor, where the membrane layer will be continuously challenged by mechanical forces (arising from scraping), will be rather limited. In contrast, PVDF and PC membranes are more robust, which is why further experiments were conducted with those two membrane variants only.

In the next step, another biofilm variant, that is created by the bacterium *B. subtilis* 3610, is cultivated on the two membranes. This biofilm variant is selected here as it has been shown to be suitable to increase the hydrophobicity of mortar as well – which is why it is of similar interest for applications in construction engineering. With the same setup and conditions as used for *B. subtilis* natto cultivation, we obtain similarly high biofilm amounts on PVDF and PC membranes as for their direct cultivation on agar (Figure 3d).

Importantly, the successful cultivation of microbial biomass is not limited to bacteria: the yeast *S. cerevisiae* can be grown on both membrane variants as well (Figure 3e); only the liquid growth medium needs to be changed to fit the needs of this particular microorganism (see Section 2 Materials and Methods). Finally, even a fungal biofilm can be cultivated on those two membranes when the growth medium and growth conditions are adjusted (see Section 2 Materials and Methods): *P. chrysogenum*, which can be employed for the production of penicillin, can be successfully grown on and harvested from both membranes using the same setup as described before (Figure 3f). In contrast, whereas cultivation of *P. chrysogenum* on bare agar surfaces works out well, it is impossible to remove the fungal biomass without destroying the agar layer (the fungus tends to grow its mycelial network into the substrate, and harvesting the fungal biomass breaks the brittle agar layer apart). Because of this problem, Figure 3f does not list a value for harvestable biomass in this particular case. This result underscores the great advantage brought about by using porous membranes as a growth substrate: they are very suitable for cultivating a broad range of different microorganisms and greatly simplify the harvesting process of the generated biomass.

### 3.3 Material properties of biofilm collected from the bioreactor

As the results described above show, the basic principle used by the bioreactor, that is cultivating microorganisms at the interface between a solid substrate and air while supplying a nutrient solution from the substrate via diffusion, is indeed suitable to grow a variety of different microorganism. Moreover, this strategy comes with the advantage of easily adjusting the type of nutrient source and harvesting the microbial biomass from the growth substrate. In previous research, bacterial biofilm – in addition to other bacterial additives (Ertelt et al., 2021) – has already been proven its value as a hydrophobizing agent in a mortar (Ertelt et al., 2020; Grumbein et al., 2016). However, the classical microbiological production process of biofilm, that is, its cultivation on sterile Petri dishes and the subsequent manual harvesting step is time-consuming and not economical. With the presented biofilm reactor, those issues are mitigated – provided that the bacterial material produced on the reactor has properties comparable to biofilm generated via the "classical" method.

To assess this, we first verify that the microbial product grown on the bioreactor is indeed composed of the bacteria of choice; this is not trivial as – different from the "classical" cultivation on agar plates – the biofilm develops on the bioreactor in a nonsterile environment. Since there are typically other bacterial spores in the air (e.g., other *Bacillus* variants than the one used for biofilm production), contamination of the reactor with other bacterial strains might compromise the quality of the grown biomass. However, as results from a contamination study conducted with serially diluted biofilm pieces collected from the bioreactor show (Figure 4), such contaminations are rare: we find mostly colonies with a morphology typical for *B. subtilis natto* colonies grown at the conditions selected here – and it is this particular strain that was used for the liquid culture (and thus biofilm growth) in those contamination tests.

We attribute this positive finding to the high number of planktonic bacteria present in the liquid culture that is used to inoculate the bioreactor and the relatively short time interval (12 h) between two individual inoculation cycles; together, those measures inhibited the growth of other, undesired bacteria on the bioreactor surface.

Accordingly, we also expect the material properties of the *B. subtilis natto* biofilms cultivated on either, agar plates or the biofilm reactor, to be very similar. We test this expectation by determining the viscoelastic properties (Figure 5a) and water content (Figure 5b) of the two biofilm variants.

Bacterial *B. subtilis natto* biofilm cultivated on agar-filled Petri dishes exhibits a viscoelastic behavior that is clearly dominated by elastic properties (Figure 5a). This is an important property for the biofilms to be successfully cultivated on the rotating biofilm reactor as it prevents the biomaterial to flow off the reactor surface during rotation. Moreover, similar to the manual harvesting process applied to collect the biofilm from agar plates, this viscoelastic material behavior enables the biofilm to be automatically collected from the cylinder by a PDMS blade. Moreover, the rheological characterization reveals that the two variants of *B. subtilis natto* biofilm show very similar absolute values of viscoelastic moduli, that is, stiffnesses in the range of $\sim 1$ kPa each (Figure 5a).

With this result in mind, it is also not surprising that both biofilm variants exhibit a very similar water content of 80%–90% (Figure 5b) – a parameter this is intimately related to the absolute stiffness of the biofilm. However, this result is not trivial: when grown in Petri dishes with the lid closed, the biofilm is very well protected from dehydration. Our results suggest that placing the biofilm into an incubator (which, in addition to covering the Petri dishes, is also done when the "classical" biofilm cultivation approach is chosen) is sufficient to control the temperature and humidity requirements during biofilm growth.

In the last step, we ask if biofilm material collected from the bioreactor is equally suitable in conveying water-repellent properties to mortar as biofilm generated on classical Petri dishes. To answer this question, we compare hybrid mortar samples containing biofilm
powder (i.e., freeze-dried bacterial biofilm) of the bacterial strain \textit{B. subtilis natto} cultivated on both, agar and the biofilm reactor, and we test the wetting resistance and capillary water uptake properties of these biofilm-enriched mortar samples (Figure 5c,d). In addition, unmodified reference mortar samples are analyzed as a control group.

To assess the wetting resistance of the samples, water contact angles are determined on the surface of small mortar samples (see Section 2 Materials and Methods). For the unmodified reference samples, low contact angles of \(\sim 35^\circ\) (Figure 5c) are obtained, and this result represents strongly hydrophilic behavior. In contrast, for biofilm-enriched samples (either obtained from cultivation on Petri dishes or from the biofilm reactor), the wetting resistance is considerably enhanced: here, we measure contact angles in the range of \(70^\circ\)–\(80^\circ\). Similarly, we obtain very good results in capillary water uptake tests (Figure 5d).
uptake tests (Figure 5d): here, the amount of invaded water is reduced from ~8.5 (measured for the control group) to ~3 g/day (measured for either variant of biofilm-enriched mortar). In other words, when mortar samples are continuously exposed to water for 24 h, both variants of the produced B. subtilis natto biofilm film can reduce the capillary water uptake of mortar by ~2/3.

### 3.4 Further possible improvements to obtain an industrial-scale bioreactor

The bioreactor we introduce here is a lab-scale prototype that fulfills its envisioned task. However, to be able to produce biomass in large amounts as relevant for industrial applications, its dimensions would need to be increased. Then, placing the bioreactor into a lab incubator will not be feasible anymore. To still provide the correct temperature levels required for biofilm growth, it should be possible to heat the hollow cylinder from the inside – and change the material of this cylinder into a material with good heat transport properties and corrosion resistance (e.g., stainless steel) will be helpful to realize this modification.

As of now, we make use of an agar layer as an intermediate structure sandwiched between the cylinder and the membrane layer; this setup is completely suitable for continuous cultivation of biofilms over several days (we here tested a given reactor configuration for up to 5 days), but it might turn out insufficient when the reactor is run for a longer period of time such as several weeks or months: since agar is a biological material, it will start to decompose at some point – and this will require a new layer to be applied to the reactor. Such a time-consuming maintenance step might be avoidable when a synthetic material is used to replace the agar layer. To guarantee a good diffusive transport of nutrients from the cylinder to the membrane layer, a hydrophilic and porous, sponge-like material is required. In addition, sufficiently high elastic properties are needed so that this intermediate layer is not squished by the mechanical scraping process used to harvest the biofilm layer. With those considerations in mind, plastic foams (made from, e.g., polyurethane, polyethylene, or polyethylene terephthalate) appear to be the most likely candidates for this purpose. These materials should be equally potent in preventing the liquid medium added to the core of the cylinder from leaking through the membrane layer while efficiently distributing the nutrients from the growth medium to the membrane surface.

When the bioreactor is used for more than several days, also toxic metabolic products that are potentially produced by the bacteria need to be taken into account. The tests conducted here, however, showed that – within the time window probed in our experiments – this is not an issue. Indeed, since the bacterial culture bath not only inoculates but also “washes” the membrane during each rotation, a strong accumulation of such toxic products is unlikely. However, within time, bacterial byproducts could accumulate in the bacterial bath, and this might hamper the reproduction of the planktonic bacteria. Thus, for continuous biofilm cultivation over a longer time period than 4 days, the bacterial culture should be regularly (ideally: continuously) replaced to maintain constant inoculation conditions and thus a constant biofilm quality. Finally, we here identified two hydrophilic membrane variants as suitable support systems to facilitate the growth of different microbial organisms. Depending on the selected microorganism, however, another membrane variant might turn out to be even more efficient. Also, it is possible that applying a macromolecular coating to the membrane can further boost the biomass production of certain microbes by promoting their attachment to the membrane layer (Ryzhkov et al., 2021; Sarjit et al., 2015), but such modifications certainly would need to be optimized for each targeted microorganism.

### 4 CONCLUSION

As we demonstrate here, a membrane-covered rotating bioreactor can be used to successfully cultivate and harvest biomass generated by different microorganisms, such as bacteria, yeast, and fungi, in a continuous manner. Importantly, the membrane layer not only provides this versatility but also allows for obtaining more biomass compared to direct cultivation on bare agar layers and ensures an easy harvesting procedure. The latter aspect is especially important when fungi are cultivated as those microorganisms tend to grow into soft substrates such as agar, and this makes harvesting them from such agar substrates very difficult. In addition to antibiotic-producing fungi such as P. chrysogenum tested here (as well as others such as Acremonium chrysogenum (Adinarayana et al., 2003), Fusidium cocci-num (Godtfredsen et al., 1979), Muscodor albus (Ezra et al., 2004; Strobel, 2006) or other endophytes (Bano et al., 2016; Shukla et al., 2014)), also other fungi such as Trichoderma harzianum (Coban & Sargin, 2019; Coşkuntuna & Özer, 2007) and Metarhizium anisopliae (Lee et al., 2005; Zimmermann, 2007) (which have applications as a biocatalyst agent in agriculture) could be interesting future targets for this bioreactor. Thus, bioproducts generated on this device could have applications in civil engineering, medicine, and agriculture.

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### CONFLICTS OF INTERESTS

The authors declare that there are no conflict of interests.

### AUTHOR CONTRIBUTIONS

Martin Kretschmer, Elif N. Hayta, Marvin J. Ertelt, and Oliver Lieleg planned the experiments, which were conducted and analyzed by Martin Kretschmer, Elif N. Hayta, Marvin J. Ertelt, and Michaela A. Würbser. The manuscript was written by contributions of all authors.
DATA AVAILABILITY STATEMENT
The authors declare that the experimental and theoretical data supporting the findings of this study are available within the paper. However, more data that support the findings of this study are available from the corresponding authors upon reasonable request.

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