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INVITED REVIEW

An overview of various methods for in vitro biofilm formation: a review

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Abstract

Biofilms are widely present in the natural environment and are difficult to remove as they are a survival strategy of microorganisms. Thus, the importance of studying biofilms is being increasingly recognized in food, medical, dental, and water quality-related industries. While research on biofilm detection methods is actively progressing, research on biofilm formation is not progressing rapidly. Moreover, there are few standardized methods because biofilm formation is affected by various factors. However, comprehensive knowledge of biofilm formation is essential to select a suitable method for research purposes. To better understand the various in vitro biofilm formation methods, the principles and characteristics of each method are explained in this review by dividing the methods into static and dynamic systems. In addition, the applications of biofilm research based on various assays are also discussed.

Keywords In vitro biofilm formation · Static method · Dynamic method · Biofilm devices · Biofilm analysis

Introduction

Biofilms are sessile microbial communities that are irreversibly attached to biotic or abiotic surfaces in nature. These sedentary complex structures are embedded in a self-produced matrix, known as extracellular polymeric substances (EPSs), consisting of proteins, non-enzymatic proteins, lipids, polysaccharides, and nucleic acids (Liu et al., 2023; Mazaheri et al., 2021). The three-dimensional structures of biofilms support and protect bacteria inside the biofilms from extreme conditions, such as heat, pH, desiccation, salinity, antibiotic use, disinfectant use, and nutritional deficiency (Van Houdt and Michiels, 2010; Yin et al., 2019). Moreover, most microorganisms, including *Bacillus cereus*, *Cronobacter sakazakii, Escherichia coli*, *Listeria monocytogenes, Pseudomonas* spp., *Staphylococcus aureus*, *Streptococcus* spp., and *Vibrio parahaemolyticus*, can form biofilms (Rewatkar and Wadher, 2013). Their biofilms can adhere to various surfaces, such as glass, rubber, stainless steel, food matrix, wood, plastic, and polypropylene, within a few minutes and can develop into mature biofilms within an hour (Carrascosa et al., 2021; Liu et al., 2023).

Biofilms are a major concern and a substantial problem in the medical and food industries because they pose a threat to public health and food safety. In the medical industry, biofilms growing on clinical devices, such as catheters and cardiac pacemakers, can cause healthcare-associated infections, resulting in patient morbidity and mortality (Percival et al., 2015). Moreover, cross-contamination due to biofilms is highly related to food poisoning (Mazaheri et al., 2021). For instance, *L. monocytogenes* were detected on various industrial surfaces and environment such as knives, gloves, tables, floor, conveyor belts, and shelves of meat retail market, cheese processing plant, cantaloupe farm, and ice cream facility. Srey et al. (2013) reported that approximately 80% of microbial infections in the United States were related to biofilms.

Biofilm-related research plays an important role in the study of microbial resistance and survival strategies (Beaudoin et al., 2012; Chen et al., 2021; Farjami et al., 2022), investigation of material properties to which cells attach (Ginige et al., 2017; Salta et al., 2018; Tran et al., 2009), and screening of antibiotics (Ersanli et al., 2023; Laverty

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et al., 2014). However, several studies have reported that the structure, biomass, and characteristics of biofilms produced under the same conditions can vary depending on the method used (Crémet et al., 2013; Hassan et al., 2011). Crémet et al. (2013) compared three different methods (crystal violet (CV) staining, BioFilm Ring Test (BRT), and resazurin assay) for assessing biofilm production. The significant correlations were observed between CV and resazurin assay ($P < 0.0001$), and between CV and BRT ($P < 0.0007$) in biofilm production. On the other hand, there was no correlation (Spearman r = 0.18; $P = 0.28$) between BRT and resazurin assay in biofilm production. When biofilm adhesion was evaluated by the tissue culture plate, tube method, Congo Red Agar method (CRA), and modified CRA method, the tissue culture plate method showed the highest biofilm production rate and formed strong biofilms (Panda et al., 2016). The variability of biofilm production according to methods suggests that methodological aspects should be considered in biofilm formation. Therefore, this review provides an overview of the most commonly used methods for in vitro biofilm research by dividing them into static and dynamic systems.

Static methods for biofilm formation

Static methods for forming biofilms are most popular in laboratory-scale experiments because of their ease of use, high producibility, controllability, low contamination, and cost-effectiveness (Cattò and Cappitelli, 2019). Particularly, these assays are useful for early-stage biofilm formation. Moreover, experiments for biofilm formation by various species can be performed simultaneously. They also have several limitations, such as the impossibility of continuous fresh medium supply and lack of aeration. Furthermore, the physiological and biological characteristics of experimentally derived biofilms are uncommon in natural environments; therefore, they cannot be compared with natural biofilms (Cattò and Cappitelli, 2019; Coenye and Nelis, 2010; Merritt et al., 2011).

Among the static methods for biofilm formation, the microtiter plate assay, Calgary biofilm device method, and BRT are the most commonly used assays for studying in vitro biofilm formation. However, biofilms can also be formed through various other methods, such as the colony biofilm assay and air–liquid interface assay.

Microtiter plate assay

A biofilm formation method using plastic tissue culture plates was first described by Fletcher and Loeb (1979). A commonly used form of this method is derived from a protocol published by Christensen et al. (1985). In this common procedure, the cell suspension is transferred to each well of a microtiter plate and incubated under specific conditions determined by the experimenter. During incubation, biofilms are formed on the bottom and the walls of the wells. After rinsing the microtiter, unattached cells are removed, leaving the formed biofilm (Fig. 1A). After biofilms are formed, the biofilms in the wells are usually stained with a dye, such as CV, for visualization. Then, the optical density (OD) is then measured using a spectrophotometer. However, other colorimetric or fluorometric assays can also be used; these include the use of tetrazolium salt derivatives, resazurin, SYTO-9 dye, and propidium iodide (Bueno, 2014; Cattò and Cappitelli, 2019; Merritt et al., 2011). For instance, Crémet et al. (2013) assessed biofilms formed by 34 *E. coli*

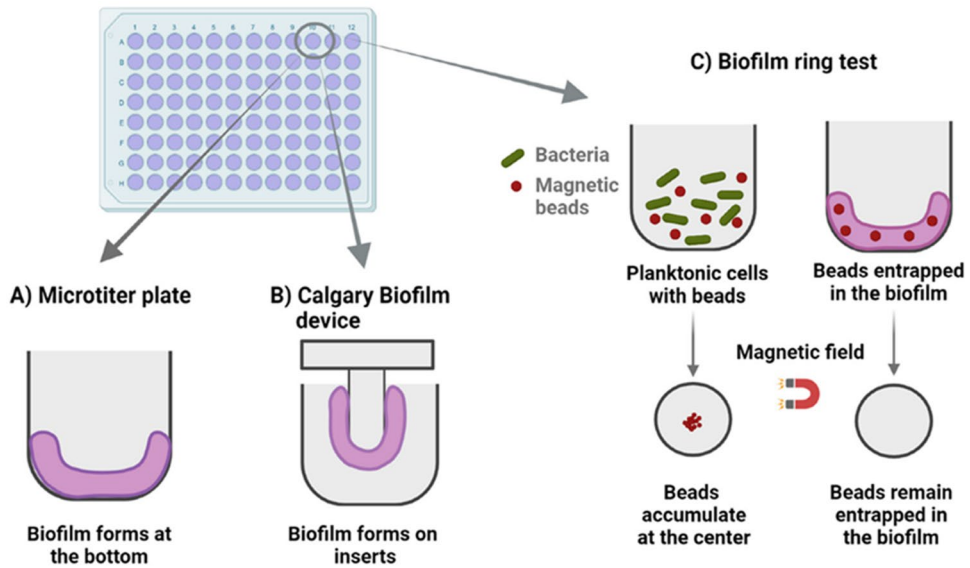

Fig. 1 Biofilm formation methods based on microtiter plates (Crivello et al., 2023). (**A**) Microtiter plate assay; (**B**) Calgary biofilm device; (**C**) BioFilm Ring Test (BRT)

isolates using resazurin staining, which is known to detect the viability of attached cells. They noted a significant correlation between CV staining and resazurin staining (Spearman $r = 0.68$; $P = 0.0001$).

In their review, Coenye and Nelis (2010) reported several advantages of this method. First, this assay is simple and inexpensive because it requires a small amount of reagents and basic experimental materials. Second, when using a 96-well plate, numerous tests can be performed simultaneously. Therefore, this method is suitable to screen biofilms formed by various strains. Finally, many variables, such as temperature and humidity, incubation time, and type of medium for biofilm formation, can be easily changed and applied to the experiments. Thus, the microtiter plate assay has been used to assess the antibiofilm activity of various antibiotics (Ersanli et al., 2023; Farjami et al., 2022; Ye et al., 2015), evaluate the effect of parameters (temperature, incubation time, nutrients, pH, and water activity) on biofilm formation (Al-kafaween et al., 2019; Han et al., 2016), and examine the features of materials to which biofilms are attached (Salta et al., 2018) (Table 1). However, the biofilms generated using this method cannot be developed into a mature form because of a lack of nutrient supply. Biofilms may also be lost in the washing step performed during staining to visualize the biofilms. Moreover, accurate biofilm observation is difficult because the biomass of dead cells or sedimented cells is also stained (Crivello et al., 2023; Magana et al., 2018).

Calgary biofilm device

The Calgary biofilm device was introduced by Ceri et al. (1999) and has been mainly used to assess the antibiotic susceptibility of biofilms (Coenye and Nelis, 2010) (Table 1). Laverty et al. (2014) used the Calgary biofilm device to investigate the antibiotic resistance of medical equipment-related pathogens (*S. epidermidis*, *S. aureus*, methicillin-resistant *S. aureus* [MRSA], *P. aeruginosa*, and *E. coli*). They found that ciprofloxacin exhibited the greatest antibiofilm activity against Gram-negative pathogens and gentamicin was most effective against Gram-positive pathogens. Another application of this device was reported by Parahitiyawa et al. (2006); they used it to standardize biofilm formation in *Candida albicans*.

The Calgary biofilm device consists of a microtiter plate and a lid, with the number of pegs being equal to the number of microtiter wells. In brief, the bacterial suspension is inoculated into the wells and the microtiter plate is incubated by covering the lid so that the pegs are submerged. Pegs are sealed and the Calgary biofilm device is incubated on a rocking table at a specific rate to generate shear forces (Ceri et al., 1999). After incubation, biofilms adhere to the pegs, while planktonic cells do not (Fig. 1B). Following biofilm formation, the lid is transferred to a second plate containing antibiotics. The upper lid is then transferred to a new microtiter plate for regrowth. The biofilm biomass or the number of sessile cells present in the biofilm can be quantified by clipping the peg on the lid (Ceri et al., 1999; Coenye and Nelis, 2010; Macia et al., 2014). This assay for biofilm formation is simple and has the advantage of being less affected by cell sedimentation than the microtiter plate assay. Also, this assay is suitable to assess late-stage biofilm formation (Azeredo et al., 2017; Renier et al., 2014). However, it is not feasible to gather individual pegs for cell enumeration (Cattò and Cappitelli, 2019). Another inherent limitation of this assay is that tightly attached biofilms are difficult to obtain by sonication. (Azeredo et al., 2017).

BioFilm ring test (BRT)

The BRT is a relatively recent method introduced by Chavant et al. (2007). They compared the BRT and the microtiter plate method with CV staining for assessing biofilm formation by *L. monocytogenes*, *E. coli*, *S. carnosus*, and *S. xylosus*. The principle of biofilm formation is based on the immobilization ability of microorganisms onto magnetic beads. In this procedure, the bacterial suspension is mixed with paramagnetic microbeads. The mixture is then loaded into a microtiter well plate. Microorganisms are attached to the paramagnetic microbeads during incubation, and biofilm formation is confirmed by applying a magnetic field. When the well of the microtiter plate contacts the magnet, free paramagnetic microbeads gather in the center of the bottom of the wells, forming a black ring. However, the biofilm-attached paramagnetic microbeads remain in place because magnetic forces are blocked (Chavant et al., 2007; Crivello et al., 2023; Magana et al., 2018) (Fig. 1C).

The predominant advantage of this assay is that it does not require washing, fixing, or staining procedures; therefore, the outcomes have low standard deviations and are less laboratory- or person-dependent for biofilm formation. Moreover, it is rapid and easy to handle. In this assay, experiments under various conditions can be performed simultaneously (Azeredo et al., 2017). In contrast, the strength of the magnetic field for determining the biofilm produced by this method may be influenced by material type and/or coating thickness. Differences in magnetic field strength between the new antibiofilm agent and its relative control may lead to analytical bias in the results. In addition, information regarding biofilm structure and thickness is not provided by this assay (Azeredo et al., 2017; Cattò and Cappitelli, 2019).

The BRT has been mainly used to quickly screen for antibacterial resistance and to determine the minimum concentration for biofilm eradication (Azeredo et al., 2017; Chavant

Table 1 Biofilm studies using static biofilm formation assays

Assay	Pathogens	Purpose	Biofilm formation conditions	Results	References
Microtiter plate assay	*S. aureus*	To screen the antimicrobial effect of essential oils	Tryptic soy broth (TSB) + 1% glucose at 37 °C for 20–24 h	All tested essential oils (*Thymus sibthorpii*, *Origanum vulgare*, *Salvia fruticosa*, and *Crithmum maritimum*) inhibited biofilm formation by 95% at half minimal inhibitory concentration (MIC)	Ersanli et al. (2023)
	E. coli	To investigate the efficacy of peracetic acid on biofilm elimination	Phosphate-buffered saline (PBS; pH 7.2) at 25–37 °C for 24–96 h	0.25% peracetic acid was effective in biofilm removal at 40 °C for more than 10 min	Farjami et al. (2022)
	V. parahaemolyticus	To examine the effect of temperature on biofilm formation	Diluted Luria–Bertani (LB) broth at different temperatures (4, 10, 15, 20, 25, 30, and 37 °C) for 72 h	A strong biofilm was formed above 15 °C, whereas a monolayer biofilm was induced below 10 °C	Han et al. (2016)
	P. aeruginosa and *S. pyogenes*	To investigate the effect of incubation time on biofilm development	Mueller Hinton (MH) broth at 37 °C for up to 7 days	Both bacteria formed the highest level of biofilms on day 3	Al-kafaween et al. (2019)
	C. sakazakii	To determine the effect of various parameters on biofilm formation	TSB at various ranges of temperature (4–44 °C), pH (3.0–9.0), and incubation time (12–48 h)	The highest biofilm formation was observed at a pH level of 5.0, temperature of 28 °C, and duration of 48 h	Ye et al. (2015)
	Cobetia marina	To investigate the effects of surface coating on biofilm development	Sea salt peptone (SSP) at 28 °C for 24 h Surface coating formula: juglone, poly(methyl methacrylate), and rosin	The thickness and biovolume of biofilms were lower on coated surfaces	Salta et al. (2018)
Calgary biofilm device	*S. epidermidis*, *S. aureus*, MRSA, *P. aeruginosa*, and *E. coli*	To screen the antimicrobial effect of antibiotics	MH broth at 37 °C for 24 h Antibiotics: vancomycin, rifampicin, trimethoprim, gentamicin, and ciprofloxacin	All antibiotics were required at the minimum biofilm eradication concentration (MBEC) for biofilm removal	Laverty et al. (2014)
	Candida spp.	To standardize biofilm formation	Yeast nitrogen base (YNB) medium supplemented with 50 mM glucose at 37 °C for 180 min for initial attachment, followed by incubation at 37 °C for 48 h	Biofilms developed over time, and sucrose induced large biomass	Parahitiyawa et al. (2006)

Table 1 (continued)

Assay	Pathogens	Purpose	Biofilm formation conditions	Results	References
BioFilm Ring Test (BRT)	*Leuconostoc mesenteroides*	To study the effect of matrix components on biofilm formation	Brain heart infusion (BHI) medium at 26 °C for 48 h Matrix components: dextran and various enzymes (pronase, proteinase K, dextranases, or nucleases)	The biofilm formation index decreased on treatment with dextran and various enzymes	Badel et al. (2008)
	P. aeruginosa	To examine the biofilm formation ability	BHI or MH broth at 37 °C up to 24 h	The BRT was found to be suitable for studying biofilm formation	Olivares et al. (2016)
Colony biofilm assay	*P. aeruginosa* and *S. aureus*	To explore the effect of organoselenium coating on cellulose disks on biofilm inhibition	Coated cellulose disk on LB agar at 37 °C for 24 h	Biofilm formation was inhibited on 0.2% selenium-coated disks	Tran et al. (2009)
	P. aeruginosa, *E. coli*, and *S. aureus*	To investigate the antibiofilm activity of slippery liquid-infused porous surfaces (SLIPS)	Porous polytetrafluoroethylene (PTFE) substrate or PTFE–SLIPS for 7 days	SLIPS inhibited the attachment of *P. aeruginosa* (99.6%), *S. aureus* (97.2%), and *E. coli* (96.0%) biofilms	Epstein et al. (2012)
Air–liquid interface assay	*P. aeruginosa*	To develop an i*n vitr*o model of biofilms on the respiratory epithelium	Diluted PBS or LB broth at 37 °C for 20 h	Biofilms were successfully formed on polarized mouse respiratory epithelium	Woodworth et al. (2008)
	P. aeruginosa	To confirm the antibacterial effect of surfactants	Lysogeny broth medium with surfactants at 37 °C for 24 h Surfactants: sodium dodecyl sulfate (SDS) and dodecyl trimethylammonium chloride (DTAC)	Trace amounts of surfactants (0.2 mM) were effective in controlling biofilm formation	Chen et al. (2021)

et al., 2007). The use of the BRT has also been expanded to study the contribution of various molecular determinants to biofilm formation mechanisms (Badel et al., 2008, 2011). Moreover, Olivares et al. (2016) used this assay to assess the biofilm formation kinetics of clinically isolated *P. aeruginosa* strains (Table 1).

Colony biofilm assay

In the colony biofilm assay, a biofilm is grown in the form of a colony on a semipermeable membrane placed on an agar medium. In brief, a sterilized membrane is placed on an agar, and bacterial culture is inoculated onto the membrane. After drying the membrane, the agar plate is incubated under appropriate conditions (Merritt et al., 2011). Although this method is simple, it is important to inoculate an equal amount of bacterial suspension onto each membrane in the first step in order to form a uniform biofilm. Table 1 presents several biofilm studies using the colony biofilm assay. Tran et al. (2009) demonstrated the efficacy of cellulose disks coated with organoselenium–methacrylate polymer in preventing biofilm formation by *P. aeruginosa* and *S. aureus*. In addition, Epstein et al. (2012) used the colony biofilm assay and reported that slippery liquid-infused porous surfaces suppressed biofilm attachment in the case of *P. aeruginosa* (99.6%), *S. aureus* (97.2%), and *E. coli* (96.0%).

In contrast, this method is difficult to handle because of the increased growth rate and colony biomass. Furthermore, as microorganisms with different surface motilities spread at different rates and biofilms are formed in different sizes, there is a limit to the simultaneous formation of biofilms by several bacterial species (Cattò and Cappitelli, 2019; Magana et al., 2018).

Air–liquid interface assay

As the air–liquid interface condition is common in nature, many biofilms grown at the liquid–solid interface are extended across and developed into the air–liquid–solid or air–liquid interface (Robertson et al., 2013). In the air–liquid interface assay, a 24-well plate is placed at an angle of 30–50° to the horizontal plane. Bacterial cultures are slowly loaded into wells of the 24-well plate such that the top edge of each culture is in the center of the bottom of the well. Then, the plate is covered with a lid and incubated for biofilm growth. The biofilm can be observed under a microscope by CV or fluorescent dye staining or using a fluorescent antibody (Merritt et al., 2011). Woodworth et al. (2008) developed an in vitro model of *P. aeruginosa* PAO1 biofilm on a polarized mouse respiratory epithelium. This assay has also been used to assess the effectiveness of anionic (sodium dodecyl sulfate [SDS]) and cationic (dodecyl trimethylammonium chloride [DTAC]) surfactants against *P. aeruginosa* (Chen et al., 2021).

This is an easy-to-perform method that requires simple equipment and allows the immediate visualization of biofilms. However, the drawbacks are that washing and staining steps are required to observe biofilms and that biofilm observation can be disturbed by planktonic cells.

Dynamic methods for biofilm formation

Dynamic methods for biofilm formation involve continuous flow systems that provide nutrients and remove waste products, allowing the biofilm to develop into a mature form over several weeks. Thus, these assays can compensate for the general limitation of static systems, which are poor nutrient supply systems (Crivello et al., 2023; Magana et al., 2018).

Typically, dynamic methods start with an adhesion step performed in a low-nutrient suspension, as the presence of a nutrient-rich suspension reduces the need for planktonic cells to adhere to the substrate (Cattò and Cappitelli, 2019; Magana et al., 2018). Then, the continuous provision of nutrients creates an environment that promotes biofilm growth on potentially antibiofilm surfaces. Therefore, the dynamic system is highly suitable for evaluating contact-killing agents, as the suspension of unattached cells is flushed out of the bioreactor after the attachment step, which allows only attached cells to develop into mature biofilms (Cattò and Cappitelli, 2019). In addition, dynamic methods allow a comparison of the effects of different media, oxygen concentrations, temperature changes, and substances on all stages of biofilm development. They can also be used in studies requiring large amounts of biofilm biomass or in studies on microsensor monitoring (Schwartz et al., 2010). On the other hand, in these methods, special devices are required to form biofilms and the experimental complexity increases. Moreover, air bubbles may be generated during flow system operation, which may disturb proper media flow and cause contamination. Another limitation is that biofilm production by various species is not possible and only a single strain can be generated per experiment (Cattò and Cappitelli, 2019; Crusz et al., 2012; Magana et al., 2018).

The representative dynamic biofilm formation assays were presented in Fig. 2. There are Kadouri system, modified Robbins device (MRD), Drip Flow Biofilm Reactor® (DFR), Rotating biofilm reactors (rotating annular reactor, rotating disk reactor, Center for Disease Control (CDC) biofilm reactor, and concentric cylinder reactor), and microfluidic-based systems.

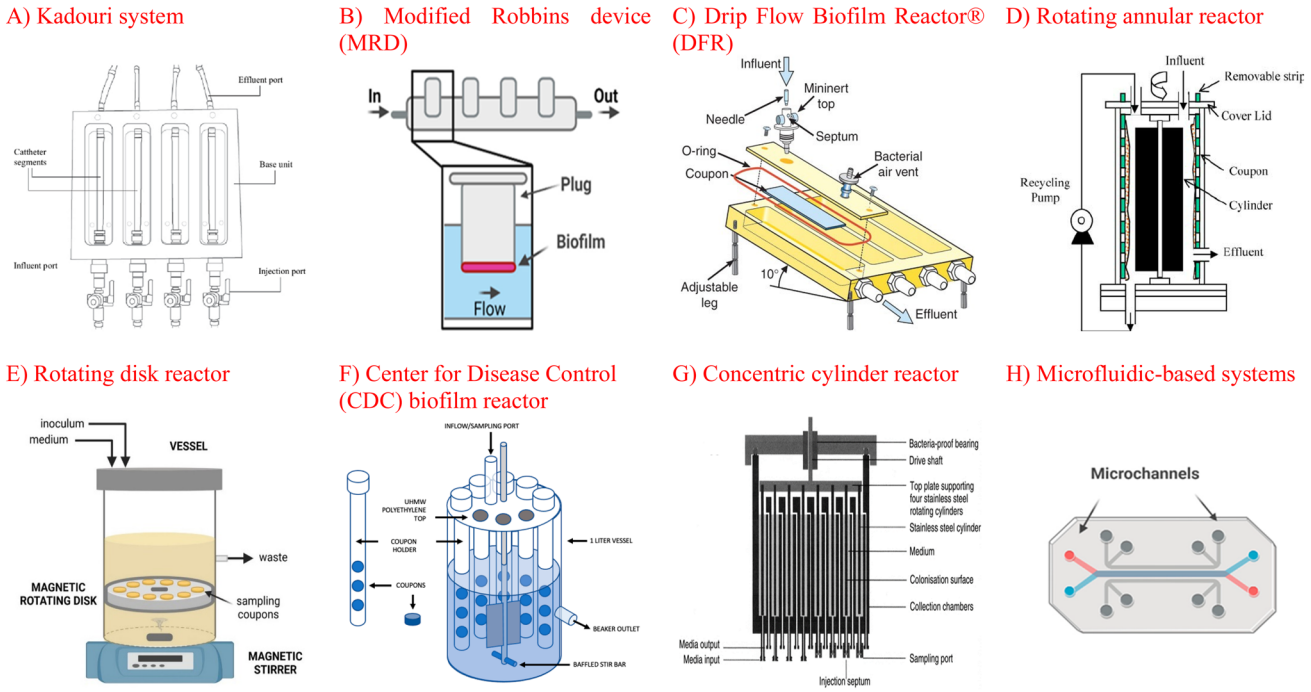

Fig. 2 Schematic diagram of various dynamic biofilm formation assays. (**A**) Kadouri system (Bueno, 2014); (**B**) modified Robbins device (MRD) (Crivello et al., 2023); (**C**) Drip Flow Biofilm Reactor® (DFR) (Goeres et al., 2009); (**D**) rotating annular reactor (Jang et al., 2006); (**E**) rotating disk reactor (Gomes and Mergulhão, 2021); (**F**) Center for Disease Control (CDC) biofilm reactor (Carrazco-Palafox et al., 2021); (**G**) concentric cylinder reactor (Willcock et al., 2000); (**H**) microfluidic-based systems (Crivello et al., 2023)

Kadouri system

The Kadouri system is a low-flow system that is intermediate between static and dynamic conditions (Merritt et al., 2011). The Kadouri system differs from the static biofilm formation assay in that it is a modified closed system with two outputs (Fig. 2A). In this system, one pump continuously supplies fresh medium, while the other removes waste and planktonic cells, developing biofilms to a mature state (Magana et al., 2018). Thus, planktonic cells in this system are not quickly swept away but can remain in the well for a longer period. The Kadoury system is commonly used in the drinking water industry to ensure water quality; its applications are described in Table 2. Jurgens et al. (2008) assessed the antibiotic resistance of *P. aeruginosa* biofilms in chloraminated drinking water. Unlike the wild type, which showed increased resistance to three antibiotics (ciprofloxacin, rifampicin, and chloramphenicol), chloraminated biofilms showed no increase in resistance to all tested antibiotics. Beaudoin et al. (2012) examined the gene expression in *ndvB*-mutant (Δ*ndvB*) *P. aeruginosa*. They found that the expression of eight ethanol oxidation genes (*ercS'*, *erbR*, *exaA*, *exaB*, *eraR*, *pqqB*, *pqqC,* and *pqqE*) decreased in Δ*ndvB* biofilms compared with the wild type, thereby inducing susceptibility to tobramycin.

This system has the strengths of the static method, such as being easy to use and supporting multiple growths by various strains. It can also generate relatively large amounts of biomass. Moreover, in this system, the developed biofilms can be directly observed under an inverted microscope (Bueno, 2014; Merritt et al., 2011). However, several papers pointed out that may occur when operating this system. The wells can dry out if media evaporates too quickly from the system. Conversely, if the flow rate of the fluid is too fast, the well may overflow. Also, colonization of the tubes through which the waste is drained can increase pressure and rupture the junctions between the tube pieces (Bueno, 2014; Magana et al., 2018).

Modified robbins device (MRD)

Originally, the Robbins device was proposed by McCoy et al. (1981) to monitor biofilm formation under various fluid velocities in a simulated situation of a drinking water facility. Then, this assay was modified to study various aspects of biofilm formation under controlled lab-scale conditions (Azeredo et al., 2017). The MRD system consists of a pipe with plugs fitted with evenly spaced coupons (Fig. 2B). Fluid flow inside the pipe can be controlled to study biofilm development under various conditions; therefore, biofilm

Table 2 Biofilm studies using dynamic biofilm formation assays

Assay	Pathogens	Purpose	Results	References
Kadouri system	*P. aeruginosa*	To screen the antibiotic resistance of biofilms exposed to chloramine	Compared with the wild type, no significant increase in resistance to antibiotics (ciprofloxacin, tobramycin, gentamicin, rifampicin, and chloramphenicol) was observed in biofilms exposed to chloramine	Jurgens et al. (2008)
	P. aeruginosa	To compare the gene expression in wild-type and *ndvB*-mutant bacterial biofilms	Ethanol oxidation-related genes decreased in *ndvB*-mutant biofilms	Beaudoin et al. (2012)
Modified Robbins device (MRD)	Natural microflora of water	To investigate biofilm formation and metal sediments depending on pipe materials	Adenosine triphosphate (ATP) and metal sediments were correlated with stainless steel and HDPE	Ginige et al. (2017)
	S. aureus and *C. parapsilosis*	To examine the antimicrobial activity of minocycline, EDTA, and 25% ethanol	The triple combination solution was most effective in biofilm diminishment	Raad et al. (2007)
	S.oralis, *Actinomyces naeslundii*, *Veillonella parvula*, *Fusobacterium nucleatum*, *P. gingivalis*, and *A. actinomycetemcomitans*	To analyze the characteristics of oral bacteria	Oral bacteria in multispecies biofilms grew over 6 log CFU/mL for 4 days, and the biofilm structure was similar to that observed in situ	Blanc et al. (2014)
Drip Flow Biofilm Reactor (DFR)	*P. aeruginosa* and MRSA	To evaluate the antibiofilm activity of lactoferrin/xylitol hydrogel	The combination of lactoferrin/xylitol hydrogel and the silver wound dressing Acticoat™ significantly reduced biofilm viability ($p < 0.05$)	Ammons et al. (2011)
	S. epidermidis, *S. aureus*, *P. aeruginosa*, and *C. albicans*	To elucidate factors affecting biofilm formation and migration in ventricular assist device drivelines	The three-dimensional structure of the driveline velour and silicone induced biofilm growth	Qu et al. (2020)
Rotating biofilm reactors	*S. epidermidis*	To investigate the effect of oxygen on biofilm formation	Biofilm development was negatively regulated by oxygen, which influenced the activity of σ^B	Cotter et al. (2009)
	S. epidermidis	To examine whether the modified rotating disk reactor assay can form biofilms under strictly limited conditions	This system was suitable for characterizing the effect of dissolved oxygen on pure cultures of *S. epidermidis*	Cotter et al. (2010)
	C. albicans	To compare two differently labeled lectins	A combination of *Erythrina crista-galli* (ECA) and *Canavalia ensiformis* (ConA) enabled better observation of biofilm EPS	Jin et al. (2005)
Microfluidic-based systems	*P. aeruginosa*	To reproduce the wound infection model	A microfluidic model was developed that could mimic the relevant physiological properties of the wound microenvironment	Wright et al. (2015)

formation can be assessed under specific hydrodynamic conditions, such as flow rate and shear stress (Crivello et al., 2023).

The general experimental method of the MRD is as follows (Hall-Stoodley et al., 1999): Cell cultures are inoculated into the MRD. Then, the peristaltic pump is turned on to ensure that the inoculated suspension moves through the MRD system to the waste container. Once the initial biofilm is formed, the system is rapidly switched to enable flow only through the medium by changing the opening and closing of the system. Moreover, the flow rate is kept low during switching to prevent liquid backflow and the MRD is tilted at a 45° angle to remove air bubbles under normal flow conditions. While this method can generate late-stage biofilms over a long period, which leads to the formation of a large biomass and is suitable for studying biofilm physiology, it requires special equipment and is more expensive than static methods. In addition, it is technically challenging. The experimenter must have prior knowledge of the flow dynamics to ensure that fluid flow is fully developed in the area where the coupon is located (Azeredo et al., 2017; Magana et al., 2018).

Ginige et al. (2017) monitored biofouling and metal sediments depending on plumbing materials (concrete, high-density polyethylene, HDPE, and stainless steel) by installing an MRD in a full-scale drinking water distribution system. They found that bacterial diversity decreased over time, but biomass and metal ion accumulation increased, especially in stainless steel and HDPE. This system has also been used in clinical industries. For instance, Raad et al. (2007) assessed the antibiofilm efficacy of minocycline, EDTA, and 25% ethanol against MRSA and *C. parapsilosis*. Blanc et al. (2014) reported another application of the MRD in oral biofilm formation (Table 2).

Drip flow biofilm reactor® (DFR)

A biofilm formation assay using the DFR was proposed by Goeres et al. (2009). The standard method is described in the American Society for Testing and Materials (ASTM) E2647-20 (2020) for *P. aeruginosa* under low shear and continuous flow conditions. The DFR consists of a rectangular base tilted at an angle of 10° and four (or six) parallel test channels where coupons are pinned (Fig. 2C). Each channel has an outflow port to allow continuous flow, a shallow trough to aid smooth flow and coupon removal at the outflow port, an effluent port to allow the escape of a continuous flow of fluid, and an alternate influent port for catheter studies. The cover contains a rubber O-ring for sealing and a valve for the bacterial air exhaust gas exchange port and inlet. The mininert valves are used to attach inoculation and media lines by matching the inlet port to each cover.

According to general instructions provided in the ASTM E2647-13, the pump flow rate and reactor angle should be set up and adjusted before the experiment. Then, the cell suspension should be passed into each chamber through a gauge needle inserted through the lid septum. The suspension inserted into the tilted DFR flows from the inlet port to the outlet port, generating biofilms (Azeredo et al., 2017; Goeres et al., 2009). Therefore, it is ideal for producing heterogeneous and gradient biofilms.

Previous studies have reported antibacterial efficacies against biofilms under weak shear conditions using the DFR system (Ammons et al., 2011; Curtin and Donlan, 2006). Qu et al. (2020) used the DFR to elucidate factors affecting biofilm formation and migration in ventricular assist device drivelines. They found that driveline infection-related pathogens attached to the soft velour section of the driveline. Moreover, the three-dimensional structure of the driveline velour and the silicone used to manufacture the driveline promoted biofilm growth (Table 2). When comparing biofilm formation data from 10 laboratories, Goeres et al. (2020) verified the reliability of the DRF, reporting 0.22 and 0.24 standard deviations in repeatability and reproducibility, respectively.

In this method, each channel can be used to form individual biofilms by various bacteria. Moreover, multiple materials can be tested simultaneously. On the other hand, difficulties in direct observation of biofilm development, low similarity with the industrial environment because of low shear stress, and a limited number of samples are the drawbacks of this method (Azeredo et al., 2017; Cattò and Cappitelli, 2019; Crivello et al., 2023; Goeres et al., 2009).

Rotating biofilm reactors

In general, the biofilm generated using rotating biofilm reactors is attached to a coupon placed in a rotating reactor or fixed material (Crivello et al., 2023). The most distinctive advantage of this system is that shear stress is generated by the rotating devices rather than the flow rate. Therefore, each parameter that causes the rotation can be independently controlled by the experimenter (Crivello et al., 2023). However, there are several disadvantages, such as the requirement for a specific device, high cost, technical challenges, and difficulty in direct observation of biofilm development (Magana et al., 2018). Another important drawback is that rotating biofilm reactors are designed in a semi-open system, which poses a risk of contamination during operation (Azeredo et al., 2017).

Rotating reactors used for biofilm formation are mainly classified into three types, namely the rotating annular reactor, rotating disk reactor, and concentric cylinder reactor, depending on the type and structure of the reactor. Thus,

as described in Table 2, many studies based on this method have been conducted in a wide range of fields (Cotter et al., 2009, 2010; Jin et al., 2005) because of the presence of various system variants.

The rotating annular reactor was devised by Kornegay and Andrews (1968). It consists of a static outer cylinder and a rotating inner cylinder (Fig. 2D). The outer cylinder is made of actual pipe material, and the inner cylinder provides a certain shear stress by controlling the rotational speed. In most annular reactors, coupons are mounted on rotating internal cylinders, except for rotating torque reactors, which are fixed on static outer cylinders (Azeredo et al., 2017). A brief description of the biofilm formation method according to the instructions provided by BioSurface Technologies Corporation is as follows:

Before the experiment, the desired operating conditions should be determined, and the rotating reactor should be set up. Then, the tested sample solution (or bacterial suspension) should be inoculated into the rotating reactor. The operating conditions should be checked routinely. During the operation, biofilms are generated on coupons mounted onto the rotating internal cylinder.

The rotating disk reactor has been described to simulate biofilm formation in a toilet environment (Pitts et al., 2001). A standard method using rotating disk reactors has been proposed in the ASTM E2196-23 (2023) for the investigation of *P. aeruginosa* biofilms under medium shear and continuous flow conditions. The rotating disk reactor consists of a disk to which several coupons are attached. It also consists of a magnet at the bottom (Fig. 2E). Rotation of the disk creates liquid surface shear across the coupons, and biofilms can be generated on the coupons. At the end of the run, the coupons can be removed from the disk and the biofilms can be scraped off the surface of the coupons for analysis and quantification. Moreover, coupons placed on different radial trajectories provide different shear stresses (Azeredo et al., 2017; Cattò and Cappitelli, 2019; Pitts et al., 2001).

An example of rotating disk reactors is the Center for Disease Control (CDC) biofilm reactor, which is a vessel with an effluent spout. The CDC biofilm reactor has eight detachable rods fixed on top of ultra-high-molecular-weight (UHMW) polyethylene, and each rod holds three detachable coupons on which biofilms can be accumulated (Coenye and Nelis, 2010) (Fig. 2F). Rotation by a magnetic stirrer provides a consistent shear force on coupons that are perpendicular to the rotating baffle and induces a continuous flow of nutrients (Cattò and Cappitelli, 2019).

The concentric cylinder reactor consists of four concentric chambers that can rotate at various speeds, dividing the inside of the reactor into four cylindrical sections (Azeredo et al., 2017) (Fig. 2G). This configuration not only allows the simultaneous testing of different surface radius-dependent shear stresses but also enables the assessment of different suspensions, as each chamber contains independent supply and sampling ports (Willcock et al., 2000). The inherent disadvantages of this reactor are that only one surface can be evaluated per experiment and the sampling process is cumbersome (Azeredo et al., 2017).

Microfluidic-based systems

Among the in vitro biofilm formation methods, the most recently proposed microfluidic-based devices are promising fluidic platforms that consist of ad hoc engineered microchannels that allow fine control of various parameters, such as nutrients, signaling molecule levels, and fluid flow conditions (Azeredo et al., 2017) (Fig. 2H). The small size of the chamber and precise operation enable close simulation of the natural environment and microscopic analysis of biofilms at single-cell resolution.

Although these devices are expensive and complex to handle, the study of biofilm formation using microfluidic-based devices is important because imaging systems and advanced software for data acquisition and analysis enable real-time observation of microbial behavior (Azeredo et al., 2017; Blanco-Cabra et al., 2021). In addition, microfluidic-based systems have a wide range of applications because they are compatible with various detection methods, such as off-chip and on-chip ones (Coenye and Nelis, 2010; Liu et al., 2023). Consequently, microfluidic-based systems compensate for the aforementioned limitations of dynamic systems, such as the requirement of knowing fluid flow dynamics, heterogeneity of biofilms, and low throughput of dynamic systems (Blanco-Cabra et al., 2021). Wright et al. (2015) developed a microfluidic model that mimics the relevant physiological properties of the wound microenvironment and assessed the changes in motility depending on the nutrient gradient (Table 2).

The most commonly used microfluidic-based methods are the BioFlux system and BiofilmChip device. The BioFlux system is a fully integrated platform that includes a 96-well plate with laminar flow, a shear motion control system, an imaging system, and software for data acquisition. In this system, the operator can adjust the shear flow value, direction, and duration using the software. In addition, fluid flow for up to 96 individual biofilms can be controlled by fine-tuning continuous or intermittent fluid flow through the pump (Cattò and Cappitelli, 2019; Magana et al., 2018). Another representative microfluidic-based system is the BiofilmChip device. The chip is designed using a combination of standard photolithography and soft lithography technologies. In this system, fluid is pumped into the BiofilmChip device through a high-precision peristaltic pump. Bacteria irreversibly adhere to the cover glass in order to create biofilms inside the chamber (Blanco-Cabra et al., 2021).

In recent decades, studies on biofilms are actively being conducted in various fields, such as food, medical, and water quality-related industries, because of bacterial contamination, infection, and equipment failure. However, the properties, physical state, and chemical structure of biofilms depend on environmental conditions and parameters. Therefore, in this review, the main in vitro biofilm formation models are described by dividing them into static and dynamic systems. In addition, the design, principle, strengths and weaknesses, characteristics, and brief protocols of each assay are discussed.

Static assays have several advantages, such as ease of handling, cost-effectiveness, less time requirement, simultaneous biofilm formation by various species, and suitability for early-stage biofilm formation and lab-scale research. Hence, static systems are appropriately used for research for screening purposes. However, the inherent disadvantages of these systems are that it is impossible to continuously supply fresh nutrients and dispose of waste. Moreover, these systems are very heterogeneous with natural environmental conditions or in vivo systems because they are closed platforms. On the other hand, dynamic assays are open systems that control important variables, such as nutrient flow and shear stress, allowing close simulation of natural environments. Consequently, mature biofilms can be observed, and large biomass can be generated. In some methods, biofilm formation can also be observed in real-time using a combination of software and imaging techniques. Although these systems can compensate for the limitations of static systems, they have some drawbacks. They are expensive because of the requirement of certain equipment. Moreover, the skill and knowledge of the operator are essential to perform the operation appropriately. It is also impossible to create multiple biofilms simultaneously in most cases.

To our knowledge, no single method fits all conditions and situations. Each method has its characteristics and drawbacks. To select a suitable assay, the following aspects should be considered before the experiment. First, the appropriate microorganisms should be selected for the research purpose. Second, the characteristics and conditions of the desired biofilm must be considered. Third, parameters should be set for biofilm formation. Nevertheless, variability exists in the results for biofilms grown under the same conditions because biofilms are living, complex, and continually evolving structures. Therefore, further research is warranted to develop simple and standardized biofilm formation procedures that can be correlated with biofilm formation noted in industrial or natural environments.

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Declarations

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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