

ORIGINAL ARTICLE

Characterization of a modified rotating disk reactor for the cultivation of *Staphylococcus epidermidis* biofilm

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Abstract

Aims: The purpose of this study was to develop a system that would allow biofilms to be cultivated under strictly defined conditions in terms of dissolved oxygen, fluid shear and to assess whether the method was suitable for the detection of respiratory activity stratification in biofilm samples.

Methods: The system is a modified version a commercially available laboratory biofilm reactor and incorporates a number of features such as the provision of defined levels of dissolved oxygen, constant average shear, enhanced gas–liquid mass transfer, aseptic operation and the ability to remove biofilm for *ex situ* analysis during or after continuous cultivation.

Conclusions: The system was shown to be effective for the characterization of the effects of dissolved oxygen on a pure culture of *Staphylococcus epidermidis*. The versatility of the system offers the potential for cultivating pure culture biofilm in defined, controlled conditions and facilitates a range of analyses that can be performed *ex situ*.

Significance and Impact of the Study: The ability to provide strict regulation of environmental conditions and enhanced transfer of oxygen to the biofilm during cultivation are important, first because oxygen is known to regulate biofilm development in several micro-organisms and second because many conventional biofilm cultivation systems may not provide adequate oxygen supply to the biofilm.

Introduction

The interaction between a biofilm and the environment in which it is grown is not completely understood. The biofilm mode of growth is believed to be a form of survival, both in an environmental or controlled laboratory setting (Donlan and Costerton 2002). From the surface of skin to a potentially anaerobic environment in the depths of an artificial implant, the bacteria that constitute a biofilm encounters a wide range of oxygen levels (Rowlinson *et al.* 2006). The majority of investigations into the influence of controlled oxygen levels on growth and antibiotic susceptibility of a biofilm have focused on *Pseudomonas aeruginosa* (Borriello *et al.* 2004; Fonseca and Sousa

2007). In *Staphylococcus epidermidis* biofilm, oxygen gradients correspond to DNA synthesis activity (Rani *et al.* 2007), and gradients in nutrient concentration have been identified as a factor in the increased susceptibility to antibiotics (Zheng and Stewart 2002). Difficulty in eradication of biofilm infection using common therapeutic means is well documented (Stewart and William Costerton 2001), but owing to a lack of tight environmental regulation in many *in vitro* systems, the influence of oxygen gradients on biofilm development and antibiotic susceptibility is unclear.

One obstacle in controlling the formation and development of a biofilm is the lack of a fundamental understanding of mechanisms associated with the formation

and effects of physiological heterogeneity. Local micro-environmental conditions in the biofilm play an important role in the behaviour of the biofilm by determining not only reaction rates but also through induction of biological responses to suboptimal conditions. Spatial physico-chemical gradients determine the local conditions in biofilms and therefore, physiological heterogeneity is inevitable. It is generally accepted that the existence of physiological heterogeneity in biofilms arises mainly as a consequence of nutrient gradients formed by the reaction-diffusion mechanism (Stewart 2003; Werner *et al.* 2004). Because of its low solubility, oxygen is frequently the limiting nutrient in biofilms. Studies have shown that oxygen limitation and consequent low metabolic activity in the interior of the biofilm are correlated with antibiotic tolerance in *Ps. aeruginosa* (Walters *et al.* 2003; Rani *et al.* 2007).

This work initially sought to characterize a biofilm reactor, known as the rotating disk reactor (RDR), commercially available from Biosurface Technologies Corp. (Bozeman, MT, USA), as a means to investigate the effect of oxygen on the growth and antibiotic susceptibility of *Staph. epidermidis* biofilm. A number of other laboratory-based systems have been used to cultivate *Staph. epidermidis* biofilm, such as the Center for Disease Control (CDC) reactor (del Pozo *et al.* 2009) and glass flow cells (Rani *et al.* 2005). The commercial RDR has been used for monoculture biofilms, as shown in the case of *Ps. aeruginosa* (Hentzer *et al.* 2001), but it can also be utilized for mixed culture samples (Zelver *et al.*, 2001). Subsequently, the RDR was developed as an American Society for Testing Materials Standard Method (Designation E2196) for the quantification of *Ps. aeruginosa* biofilm grown in shear and continuous conditions. Table 1 summarizes the details of published studies where the commercial RDR was used to produce biofilm. A second phase of this work was to develop and characterize a modified RDR system that was designed to provide improved gas-liquid mass transfer and mixing. The primary objective of this part of the study was to investigate whether the modified RDR would allow biofilms to be cultivated under strictly defined conditions in terms of oxygen levels, fluid shear and aseptic operation and to assess whether the method was suitable for the detection of respiratory activity stratification in the biofilm.

Materials and methods

Bacterial strains and media

Staphylococcus epidermidis 1457 (Mack *et al.* 1992), a known strongly adherent biofilm producing strain, was stored in protect beads at -80°C and revived in 50 ml

brain heart infusion (BHI; Oxoid, Basingstoke, Hampshire, UK) broth overnight in an incubated orbital shaker at 37°C and 150 rev min^{-1} without aeration (Holland *et al.* 2008). This strain was a kind gift from Dietrich Mack to the O'Gara laboratory.

The rotating disk biofilm reactor (RDR)

Both the commercial rotating disk reactor (designated as cRDR) and a modified rotating disk reactor (mRDR) were used in this study. The cRDR; (Biosurface Technologies Corp) consists of a 1-l glass vessel with a waste outlet located one-quarter of the height (*c.* 40 mm) from the bottom of the reactor. This provides a working volume of *c.* 180 ml when the disk is in place and rotating; though, this reduces with increasing speeds of rotation because of vortex formation. The lid has an inoculation port and three ports for liquid medium or gas input. The RDR can be operated in batch or continuous flow, and the velocity field under which the biofilm is formed is adjustable by means of the magnetically driven rotating disk (diameter 70 mm) that sits at the bottom of the reactor vessel. All rotational speeds reported were verified using a tachometer prior to the initiation of operation. Rotation of the disk provides continuous mixing in the system. The disk holds six coupons in place (diameter 12.7 mm), which are inserted prior to autoclaving. In these experiments, polycarbonate coupons were used. The disk is removed by a sterile hook when the biofilm is to be analysed. The entire system, without the peristaltic pump, was autoclaved prior to use at 121°C for 15 min. The reactor and feed were placed in an incubator at 37°C .

Modified RDR (mRDR)

The commercial RDR was modified to facilitate the input of gas directly into the liquid media. A stainless steel sparger (inner diameter 4 mm, outer diameter 7 mm) extended the gas input port into the liquid, allowing gas to be sparged directly into the liquid. The sparger was widened at the bottom (to 15 mm, for the bottom 19 mm of the pipe) to simultaneously act as a baffle in the system, reducing the vortex formed when the disk rotates. The lengths of the sparger were specified so that if gas was input through it, bubbles would not interfere with the rotation of, or scour any biofilm adhering to, the surface of the disk. The mRDR and cRDR are shown schematically in Fig. 1.

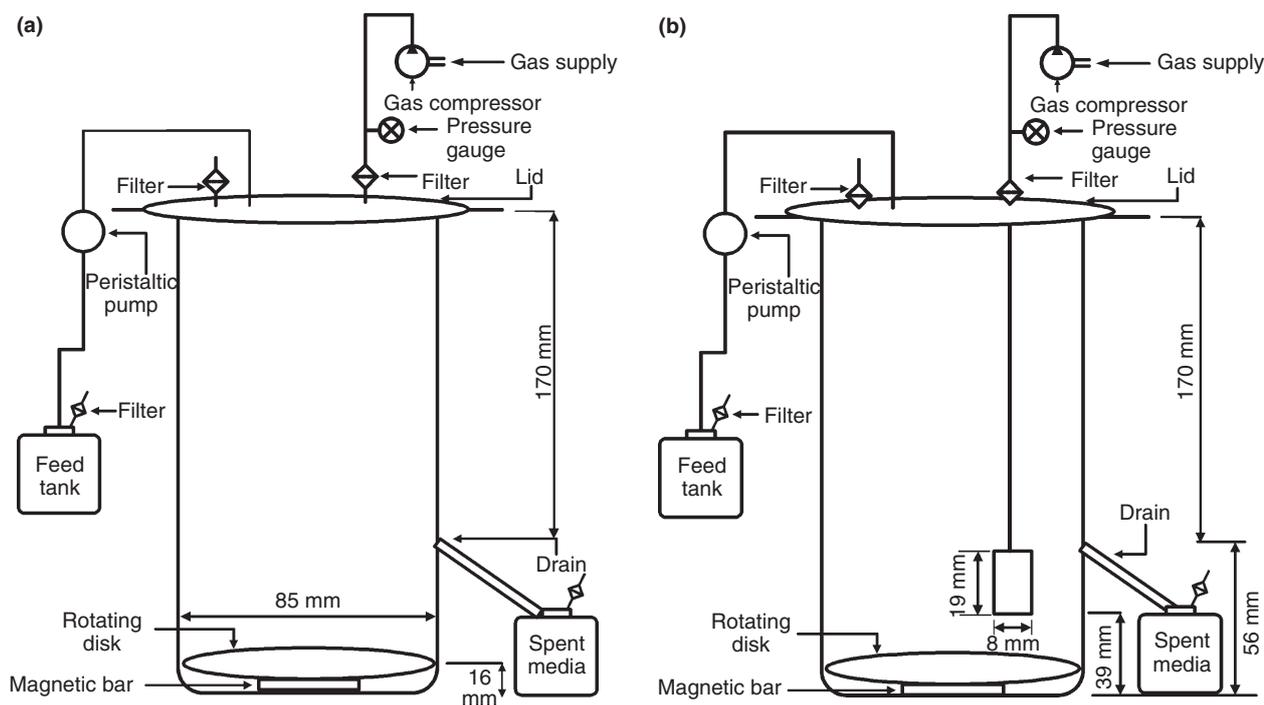
Cultivation procedure applied to both cRDR and mRDR

The reactors were filled with BHI and sterilized by autoclaving and subsequently placed in an incubator at

Table 1 Summary of some published studies where the commercial rotating disk reactor was used to produce biofilm

Organism	Operational mode	Rev min ⁻¹	Gas input	Source (year)
Mixed	Continuous	50	None	Khan <i>et al.</i> (2010)
<i>Pseudomonas aeruginosa</i>	Continuous	200	None	Garo <i>et al.</i> (2007)
<i>Ps. aeruginosa</i>	Continuous	N/A	None	Banin <i>et al.</i> (2006)
<i>Ps. aeruginosa</i>	Batch	N/A	None	Boles <i>et al.</i> (2004)
<i>Streptococcus mutans</i>	Continuous	100	CO ₂ filled headspace	Vinogradov <i>et al.</i> (2004)
<i>Ps. aeruginosa</i>	Continuous	N/A	None	Teitzel and Parsek (2003)
<i>Staphylococcus aureus</i>	Continuous	N/A	None	Yarwood and Schlievert (2003)
<i>Ps. aeruginosa</i>	Continuous	N/A	None	Singh <i>et al.</i> (2002)
<i>Ps. aeruginosa</i>	Continuous	N/A	None	Hentzer <i>et al.</i> (2001)
<i>Ps. aeruginosa</i>	Continuous	N/A	N/A	Zelver <i>et al.</i> (1999)
Mixed	Continuous	N/A	N ₂ into headspace*	Zelver <i>et al.</i> (1999)

*In this paper the nitrogen is sparged into the bulk feed medium, creating a positive pressure forcing the gas into the headspace of the reactor.

**Figure 1** Schematics of (a) commercial and (b) modified rotating disk reactor in continuous mode. Dimensions are recorded in mm.

$37 \pm 1^\circ\text{C}$. One hundred and fifty millilitres of liquid BHI was used for the cRDR reactors, with 180 ml used for mRDRs. The difference in this starting volume relates to the influence the vortex has on the liquid volume in the reactor and is explained in the Discussion

section. A 1% (v/v) solution of an overnight culture (adjusted to $A_{660} = 1.0$) was used to inoculate each reactor. This concentration of cells equated to $c. 10^5$ CFU ml⁻¹. For continuous operation, quarter-strength BHI was drip fed from the lid into the reactor.

Using a small modification to the tubing on the waste line, samples of planktonic cells were removed for the quantification over time. Samples were taken throughout a 48-h run with offline optical density measurements at 660 nm (OD_{660nm}) and colony forming units (CFUs) assessed at each time point.

Gas of known oxygen concentrations was sparged into mRDR. Nitrogen and air (Air Products, Dublin, Ireland) were blended in a gas mixing station, with oxygen concentration verification performed offline using a gas analyser prior to the commencement of every run. Gas was sparged at 0.5 l min^{-1} throughout all runs. Concentrations of 0, 7, 14 and 21% oxygen were examined for their influence on biofilm formation. Most comparisons were made between the 0 and 21% oxygen-sparged biofilms.

Monitoring of dissolved oxygen

As a means of assessing oxygen utilization in the modified reactor by *Staph. epidermidis* 1457, fluorometric oxygen sensor spots (PreSens GmbH, Regensburg, Germany) were used to record the dissolved oxygen levels within the RDR during experiments where the sparged gas contained 0 and 21% oxygen. The sensor spots (Puskeiler *et al.* 2005) were adhered to the inner wall of the RDR between the air-liquid interface and the disk using a minimal amount of silicone rubber compound 692-542 (RS Components, Corby, UK) and transmitted readings to a detector outside of the reactor. This method to measure oxygen via dynamic fluorescence quenching allows *in situ* measurement of dissolved oxygen concentrations in aqueous solutions. The spots, which have an area of 28 mm^2 , are designed to operate through glass or any transparent substance and were sterilized in place as the reactor was autoclaved. The accuracy of the spots is $\pm 0.4\% \text{ O}_2$ at $20.9\% \text{ O}_2$ and $\pm 0.05\% \text{ O}_2$ at $0.2\% \text{ O}_2$ (Liebsch *et al.* 2000). The range of detection is $0\text{--}45 \text{ mg l}^{-1}$.

Volumetric mass transfer calculations for the cRDR and mRDR

To calculate the value for volumetric mass transfer in the cRDR and mRDR, nitrogen was pumped into each reactor under normal operating conditions (37°C) using sterilized H_2O as media and without bacterial inoculation. If a near saturated liquid mass is contacted with an oxygen-free gas, e.g. pure nitrogen, then desorption will occur with oxygen transferring from the oxygen-rich liquid to the sparging gas. An oxygen balance on the liquid for a short time dt yields;

$$-d(\text{VC}) = k_{\ell}a\text{VC}dt$$

where a refers to the interfacial area per unit volume of liquid. Integration of the earlier equation between time $t = 0$ and time $t = t$, when the oxygen concentrations are C_0 (often zero) and C_t , respectively, yields,

$$\ln \frac{C_0}{C_t} = k_{\ell}at$$

a semi-log plot will yield a value for $k_{\ell}a$.

Quantification of biofilm

The variation of biofilm CFUs adhering to coupons in the same reactor and coupons in different reactors under identical conditions was assessed. Six coupon spaces within one rotating disk ensure that the repeatability of biofilm formation within one reactor can be easily analysed. To compare biofilm formation under the different reactors, each type of RDR was run in one incubator with the same inoculum, media and disk rotational speed.

To quantify biofilm adherence, coupons from two cRDRs and two from mRDRs, thus 12 coupons from each type of reactor, were removed from the reactor, washed and placed in 9-ml sterile Ringers (Hall-Stoodley and Lappin-Scott 1998). For the sonication method, the coupons in Ringers were vortexed for 5 min, sonicated for 2 min and then vortexed again for 2 min. The sonication was designed to lift the biofilm from the surface of the coupon, and the vortexing was optimized to provide maximum disruption of the aggregates to create a uniform solution that could be serially diluted and plated on nutrient agar to assess the CFUs. Plates were incubated at 37°C for 24 h. Statistical comparisons were made using the Student's t -test and CFU calculations according to Zilver *et al.* (1999).

Influence of disk rotational speed on biofilm formation

Modifying the operation of a cRDR to produce biofilm under different shear conditions relies on the interaction between the disk and the magnetic stirrer. Three rotational speed settings were examined for *Staph. epidermidis* biofilm formation: low (100 rev min^{-1}), medium (150 rev min^{-1}) and high (200 rev min^{-1}) settings. The Reynolds numbers were 8167, 12 250 and 16 333 for the rotational speeds 100, 150 and 200 rev min^{-1} , respectively. All rotational speeds were verified prior to inoculation using a tachometer (Tm-3011 tachometer; Veeder Root, Richmond, UK). Experiments where the rotational speed was above 200 rev min^{-1} are not reported because

the unstable nature of the disk rotation over an extended period of time, such as 24 h, frequently led to the disk stopping before the conclusion of operation. The interaction between the magnetic stirrer and the disk is a limitation of this system, which requires careful consideration prior to selecting a rotational speed.

Antibiotic susceptibility of *Staphylococcus epidermidis* biofilm grown under different oxygen concentrations

In these experiments, rifampicin treatment of biofilm samples was analysed for log reduction in CFU counts after 24 h of treatment. Samples were removed from the RDR after 48-h growth in different sparged oxygen conditions and treated with 2, 1 and 0.5 $\mu\text{g ml}^{-1}$ for 24 h at 37°C. These experiments were performed to investigate whether the sparged oxygen concentration in which the biofilm was cultivated had an influence on the antibiotic susceptibility of the cells.

Application of fluorescent stains

For mRDR coupons, biofilms were washed three times in Ringers to remove any planktonic cells. Samples were stained with 5-cyano-2, 3-ditoyl tetrazolium chloride (CTC), a tetrazolium salt that is reduced to a fluorescent derivative indicating redox activity in a biofilm. It has previously been used as an indicator of respiratory activity in bacteria (Rabinovitch and Stewart 2006), and a final concentration of 0.04% in deionized autoclaved water was applied directly to the coupon, prior to incubation at 37°C for 1 h in the dark (Yu and McFeters 1994). Samples were dipped in sterile Ringers after this time. For this method, as with all the analysis methods used in these experiments, the biofilms adhered to the coupons were sacrificed and were not used for other analyses. These experiments were only performed on mRDR biofilms as the oxygen levels in which the biofilm was cultured was known, rather than in the cRDR where no attempt is made to control the environment.

Cryoembedding biofilms

To prevent the damage occurring to a sample exposed to freezing conditions, biofilms were embedded in a protective medium (O.C.T. Tissue-tek embedding compound, Sakura, Zoetenwoude, the Netherlands) for 10 min after staining. This allows the O.C.T. time for complete penetration of the biofilm and maximum protection. For coupon samples, the embedded biofilm is placed into a chamber of containing *n*-hexane surrounded by liquid carbon dioxide for 10 min according to the procedure outlined by Fox *et al.* (1995). Biofilms were stored at

−80°C prior to sectioning. Biofilms were stored in triplicate for each of the subsequent analyses.

Cryosectioning

Cryosectioning was performed at −23°C (Yu *et al.* 1994) in a Microm HM500 (Microm, Walldorf, Germany). For biofilms attached to coupons, the substratum was removed prior to the biofilm being mounted on the slicing stage. The samples were positioned to enable a cross-section of the biofilm to be taken. Slices of 5 μm were cut and stored on poly-L-lysine-coated slides prior to counterstaining with 100 $\mu\text{g ml}^{-1}$ of 4',6-diamidino-2-phenylindole (DAPI) in the dark for 3 min. Poly-L-lysine is used to create an attraction between the slides and the sample, and in this method DAPI is a counterstain to the previously used CTC, which indicates the areas of respiratory activity only. Slides were viewed using an Olympus BX51 fluorescent microscope (Olympus, Tokyo, Japan) at 40× magnification. Fluorescent images were overlaid using Adobe Photoshop (Adobe Systems, San Jose, CA, USA), and thickness measurements were taken using Able Image Analyser (Mu Labs, Ljubljana, Slovenia).

Results

Influence of disk rotational speed on biofilm formation

Three rotational speed settings were examined for *Staph. epidermidis* biofilm formation in the commercial RDR, with an inversely proportional relationship identified between rotational speed and biofilm quantity. The greatest biofilm accumulation occurred at the lowest speed examined, 100 rev min^{-1} , where $9.16 \pm 0.2 \log \text{CFU cm}^{-2}$ of biofilm adhered to the coupons. Experiments at 150 rev min^{-1} gave $8.05 \pm 0.31 \log \text{CFU cm}^{-2}$ biofilm and 200 rev min^{-1} gave $7.66 \pm 0.35 \log \text{CFU cm}^{-2}$. Fonseca and Sousa (2007) noted that shear had a negative effect on biofilm formation in *Ps. aeruginosa*, which correlates with our results. For the purposes of maintaining consistency in subsequent investigations, 200 rev min^{-1} was chosen as the rotational speed of the disk. Although the exact value of shear encountered in *in vivo* biofilms is unknown, it is known that biofilms in, for example, artificial implants do not necessarily encounter static conditions (Costerton *et al.* 2007).

Biofilm formation in anaerobic atmospheric conditions

To examine the influence anaerobic conditions have on *Staph. epidermidis* biofilm formation, filtered nitrogen gas (Air Products) was pumped into the headspace of the

cRDR at 0.5 l ml^{-1} for the duration of a 24-h batch run. Biofilm formed in greater amounts with nitrogen pumped into the headspace, with $8.31 \pm 0.19 \text{ log CFU cm}^{-2}$ biofilm forming on coupons in anaerobic conditions compared with $7.65 \pm 0.14 \text{ log CFU cm}^{-2}$ biofilm forming on coupons in non-aerated conditions. This is a statistically significant difference in biofilm formation on the coupons in terms of culturable cells ($P < 0.05$). Biofilm formation occurred at the interface between the disk, liquid and headspace (not shown), and this accumulation was a factor in the construction of the sparger with a widened base to alter the hydrodynamic conditions and reduce the size of the vortex formed by the rotation of the disk.

Biofilm formation under various sparged oxygen conditions in the mRDR

To quantify planktonic cell growth, samples were taken throughout a 48-h run and analysed for OD and viable cells. Figure 2a shows planktonic growth for a nitrogen-sparged system, and Fig. 2b for a 21% oxygen-sparged system. The CFU counts for both are similar, particularly when the oxygen is consumed in the 21% sparged oxygen reactors (Fig. 2b), but the optical density of the planktonic liquid is greater for the 21% oxygen reactors. In a dynamic system such as the modified RDR, attachment and reattachment of biofilm is expected to be ongoing throughout the duration of a run, but with biofilm formation greater under anaerobic conditions, this may explain why the OD in nitrogen-sparged conditions is so much lower than that in 21% oxygen conditions.

Oxygen mass transfer rate measurements

Using the sensor spots to assess dissolved oxygen in the reactor under abiotic conditions, calculations of the volumetric transfer of oxygen under different rotational speeds were performed for the cRDR and mRDR (Table 2). The modifications to input gas directly into the media in the mRDR have a considerable effect on the transfer of oxygen into the media compared with the cRDR, with a tenfold difference in volumetric mass transfer at 200 rev min^{-1} .

Dissolved oxygen concentration in liquid

Sensor spots on the inner wall of the modified reactor were positioned to give direct readings of dissolved oxygen from the liquid medium. Figure 3 displays the profiles for 0, 21 and 100% sparged oxygen. Profiles are shown up to 24 h and are the mean of two independent runs. Oxygen concentration at saturation was c .

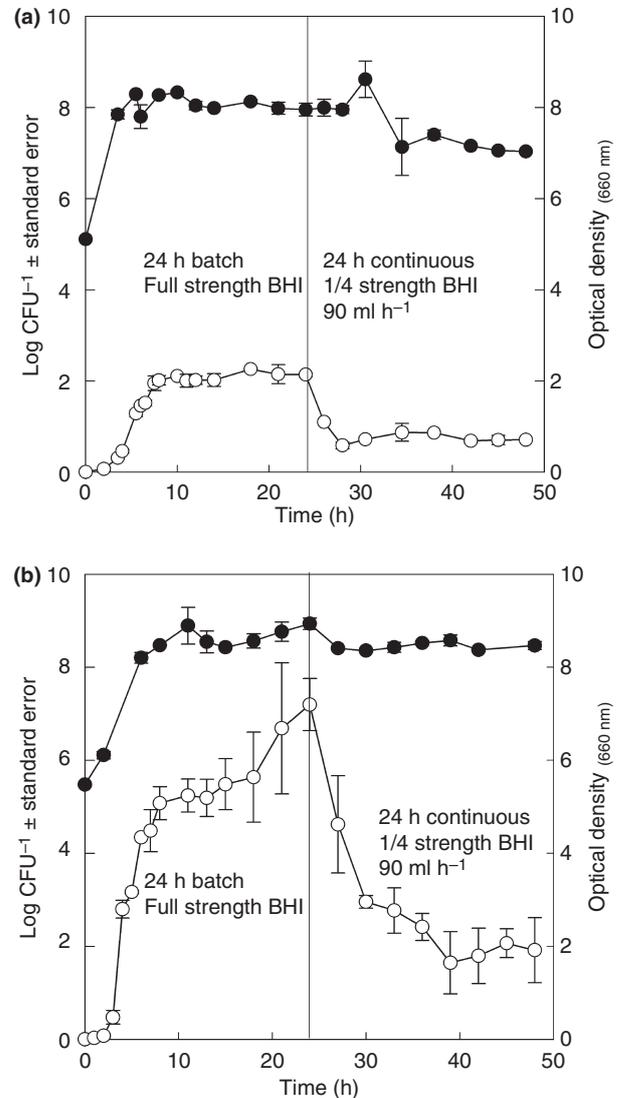


Figure 2 Planktonic cells of *Staphylococcus epidermidis* 1457 taken from a modified rotating disk reactor sparged with (a) 0% oxygen and (b) 21% oxygen at a flow rate of 0.5 l h^{-1} . Quarter-strength media was supplied at the rate of 90 ml h^{-1} . Log CFU ml^{-1} (●) and optical density (660 nm) (○) are the mean of three independent reactors.

Table 2 Volumetric mass transfer coefficients in the modified rotating disk reactor (mRDR) and commercial rotating disk reactor (cRDR) at different rotational speeds

Speed (rev min^{-1})	$k_L a$ (h^{-1})	
	mRDR	cRDR
150	0.204	0.0210
200	0.352	0.0323
300	0.950	0.0411

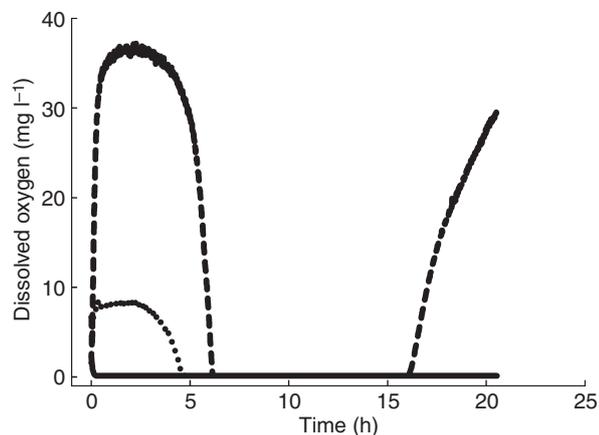


Figure 3 Dissolved oxygen profiles for *Staphylococcus epidermidis* 1457 in the liquid phase grown in a modified rotating disk reactor sparged with different oxygen concentrations. Profiles are a mean of two independent runs. (—) 0% sparged oxygen; (.....) 21% sparged oxygen and (- - -) 100% sparged oxygen.

38 mg l⁻¹. The dissolved oxygen for the 0% sparged oxygen drops below the detection within 30 min, and the 21% sparged oxygen drops below the detection after 4.5 h.

To examine the extent of oxygen limitation in the system, 100% oxygen was sparged into the reactor. The profile shows that despite the use of pure oxygen in the gas supplied and the enhanced rate of oxygen supply in the mRDR, the demand for oxygen exceeded the rate of supply under these conditions, and the dissolved oxygen drops below the detection after 7 h. From 16 h onwards, as the growth rate of the *Staph. epidermidis* cells begins to reduce and they enter stationary phase where they consume less oxygen, the dissolved oxygen profile begins to rise to c. 35 mg l⁻¹ again when oxygen demand diminishes.

Cryoslicing of biofilms cultivated under different oxygen concentrations

Biofilms stained with CTC and DAPI were examined at 40× magnification. Biofilms cultured in oxygen concentrations of 0, 7 and 21% are displayed in Fig. 4. Thickness measurements are included in Fig. 5. Overall coverage of the coupon surface with biofilm cultivated in 0% oxygen was greater than that of biofilm grown in 21% oxygen, and a statistical difference is noted ($P < 0.05$). Similar results were reported for biofilm viable cell counts under these conditions when assessed by CFUs after the biofilm was removed from the surface of the coupon. These images were acquired with the intention of investigating whether gradients in respiratory activity occurred between the biofilms grown in different oxygen conditions. To

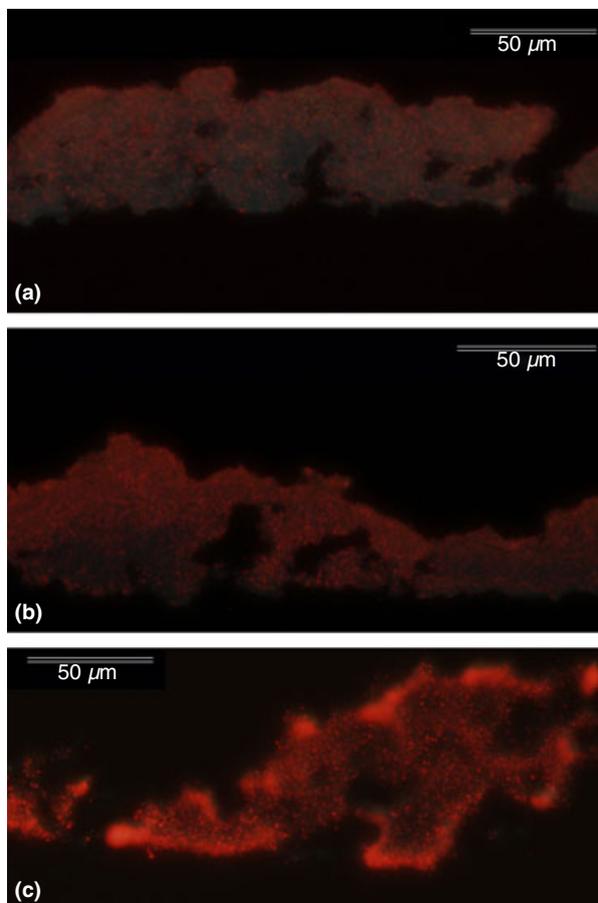


Figure 4 Overlaid fluorescent images of biofilm cryoslices grown in a modified rotating disk reactor with sparged gas of different oxygen concentrations. Slices of 5 μm thickness were stained with 5-cyano-2,3-ditolyl tetrazolium chloride and 6-diamidino-2-phenylindole and imaged at 40× magnification. The coupon was located at the bottom of these images before the removal prior to slicing. Bar = 50 μm. (a) Biofilm from 0% oxygen environment. (b) Biofilm from 7% oxygen environment. (c) Biofilm from 21% oxygen environment.

perform this, any biofilm that could be sliced from the surface of the coupon was used, and thus these pictures give a narrow field of view on the entire biofilm forming on the coupon. However, from what is displayed in these photos, activity gradients are not apparent, with CTC evenly distributed throughout the image.

Antibiotic susceptibility of biofilm cultivated under different oxygen concentrations

The mRDR can be utilized as a reactor for producing monoculture biofilm, under different oxygen concentrations, for susceptibility testing and thickness measurements. In these experiments, rifampicin treatment of biofilm samples grown in the RDR for 48 h in different

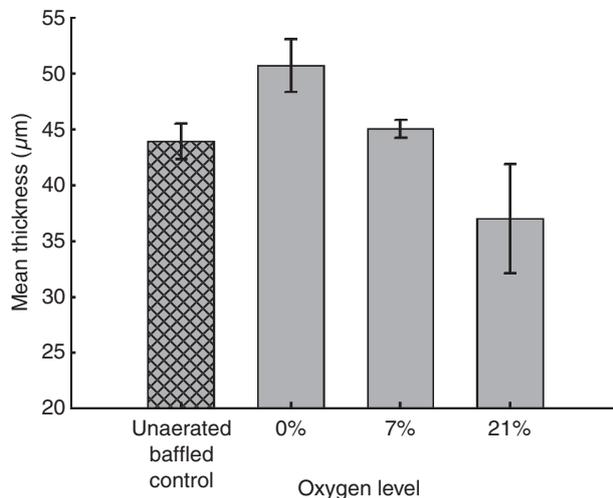


Figure 5 Mean thickness measurements from *Staphylococcus epidermidis* 1457 biofilm grown in a modified rotating disk reactor under different sparged oxygen concentrations. Error bars are standard error of the mean of two slices of three biofilms, measured 12 times for thickness.

sparged oxygen conditions was analysed for CFU log reduction after 24 h of treatment. All concentrations of rifampicin tested showed a reduction in viable cells and a reduction in thickness. For the highest concentration of rifampicin tested ($2 \mu\text{g ml}^{-1}$), biofilm log CFU cm^{-2} counts in 0% sparged oxygen conditions fell from 8.9 prior to treatment to 7.56 post-treatment. In 21% sparged oxygen biofilm samples, log CFU cm^{-2} counts fell from 8.57 prior to treatment to 7.02 post-treatment.

Discussion

Commercial rotating disk reactor (cRDR)

The majority of biofilm reactors available are designed either for reproduction of biofilm or for visualizing the adherence of cells (McClellan *et al.* 2004). The RDR is a novel system and is shown here to be suitable for producing monoculture staphylococcal biofilm for a variety of investigations. Like other commercial bioreactors, the RDR can be used for a variety of purposes, and with the additional applications of cryoembedding and cryoslicing, it can produce six biofilm samples adhered to removable coupons that can be quantified or sectioned for microscopy. The RDR can also be used to harvest cells for RNA extraction, which can enable the detailed analysis of genetic changes in biofilms in response to imposed environmental conditions as discussed previously (Cotter *et al.* 2009). For this work the authors found the RDR easier to

modify than the CDC reactor, and preferable to glass flow cells as biofilm was easier to extract and quantify.

The RDR is completely autoclavable and therefore allows for the cultivation of pure culture biofilms on easily removable coupons for *ex situ* analysis. To enable the assessment of viable cells, biofilm was removed by sonication. The design of the RDR disk, which holds the coupons on place, allows the contact between the nutrient media and both sides of the coupon. Scraping both the top and bottom of coupons showed no increase in biofilm CFUs compared with scraping the top only, and we conclude that the biofilm forming on the bottom of the coupons is minimal. This may be attributed to the design of the disk in which the bottom of the coupon is exposed to the media but is not flush with the bottom of the rotating disk. This may play a role in reducing the biofilm formation on the underside of the coupon. This discovery resulted in only the top surface of the coupon being taken into consideration in quantifying biofilm CFU cm^{-2} .

Pumping nitrogen gas into the headspace of a cRDR showed greater biofilm adherence at the interface between the vortex and the disk. A thick, wide band of biofilm that accumulated at this intersection (not shown) is accounted for by the sideways rocking motion that the disk experiences when it rotates at higher speeds. This accumulation band did not correspond with the positioning of the coupon surfaces and had no bearing on the CFU counts reported.

Comparison of batch and continuous RDR operations must take into account the change in liquid volume because of the influence of the vortex. The positioning of the effluent port (Fig. 1), which is closed for batch operations, can become a factor with a larger vortex. In continuous operations, the feed volume that needs to be added prior to the liquid volume reaching the height of the effluent port, thus creating continuous conditions, will vary depending on the speed of the disks rotation. This speed differential dictates the height reached by the vortex and is an important consideration for future work using this reactor.

The rotational speed of the disk had little observed effect on the volumetric mass transfer coefficients of oxygen ($k_L a$) values in the cRDR as increasing the rotational speed of the disk does not alter the size of the interface between the vortex and reactor headspace. In contrast, there is a significant effect of rotational speed on $k_L a$ values in the mRDR where the sparger acts as a baffle and the mixing is enhanced. Rotational speed also influences shear at the liquid biofilm interface. The effect of shear on biofilm structure is well documented in different systems (Stoodley *et al.* 1999a,b; Tsai 2005). Although the quantification of biofilm structure was not an objective of

the present work, the RDR can be expected to be a suitable system for the study of the effect of shear on biofilm structure. Greater rotational speed increases oxygen transfer rate and reduces biofilm formation; these results correlate with previously published data identifying that higher oxygen levels reduce biofilm formation via the repression of *icaADBC* operon in *Staph. epidermidis*, which controls the production of polysaccharide intercellular adhesin (PIA) (Cotter *et al.* 2009). However, choice of rotational speed in laboratory RDRs should be made based on other practical constraints such as the prevention of an excessive vortex that may result in hydrodynamic conditions adjacent to the biofilm resulting in possible detachment.

Modified rotating disk reactor (mRDR)

The modifications to the RDR methodology used a constant stream of blended gas through a sparger to accurately control oxygen concentrations within the reactor and media. An important advantage of the RDR is that, unlike flow cells, the hydrodynamic conditions in the bulk liquid surrounding the biofilm do not vary significantly during the course of biofilm development. The presence of the sparger caused the vortex to migrate to the free surface of the media, and although a shallow vortex was still present, it had no interaction with the surface of the disk. With the vortex all but eliminated, the difference in the liquid volume between batch and continuous operations was also negligible, with a volume of 180 ml remaining constant after the initiation of feed. The sparger was designed to add oxygen to the liquid at a higher rate than in the cRDR; moreover, it served to eliminate this vortex-disk interaction and distribute the biofilm more evenly across the surface of the disk. Direct sparging into the media increased the mass transfer of the gas into the liquid by tenfold compared with the cRDR, and biofilm CFUs were increased further when nitrogen was sparged into mRDR compared with the headspace being pumped with nitrogen in the cRDR. Results in Table 2 show a moderate increase in the k_{La} with increased rotational speed in the cRDR, as the vortex size determines the rate of oxygen transfer from the headspace into the liquid. In contrast, increased rotational speed has a significant positive effect on mass transfer in the mRDR where the k_{La} is determined primarily by the dispersion of bubbles from the sparged gas. Thus, the potential for strict regulation of environmental conditions and better mass transfer of oxygen in the mRDR indicates the obvious advantages in using this reactor for the purposes of growing biofilm under defined conditions. When comparing the mRDR k_{La} values with a stirred tank bioreactor, the deficiency in mixing within the mRDR is obvious. But for

overall convenience in extracting biofilm for a range of purposes, the system is an important option for *in vitro* studies and could be used in future for similar aerobic and anaerobic investigations using a range of medically or environmentally relevant bacteria. The cells adhering to the coupons still undergo the same shear forces irrespective of the action of the sparger.

The most important and commonly used coupon-based biofilm production system alternative to the RDR is the CDC reactor, also commercially available from Biosurface Technologies. The CDC reactor incorporates 18 coupons that sit into six removable rods (Williams and Bloebaum 2010). The input of sparged gas is not an option. In the CDC reactor, the coupons are static with a stirrer used to create shear on the surface of the coupons. This lack of environmental control and difference in coupon quantity are some of few differences between the reactors, as both systems are capable of being used to produce monoculture biofilm adhered to coupons for a variety of analyses.

Dissolved oxygen readings from the RDR

Dissolved oxygen in a biofilm system was measured by noninvasive sensor spots that have, to our knowledge, not previously been used in biofilm reactors. The sensor spots have previously been used in shake flask cultures for both microbial and mammalian cell cultures (Puskeiler *et al.* 2005; Naciri *et al.* 2008). There is a large potential for use in quantifying dissolved oxygen in glass biofilm reactors, as our investigations focusing on biofilm formation on the surface of the spots, which could interfere with the fluorescent quenching system, showed that cell adherence was not at a significant level to affect the readings. Cells did adhere to the spots, but the level of adherence was significantly lower than the quantity of biofilm adhering to the coupons on the rotating disk. Dissolved oxygen measurement in the nutrient media in the mRDR demonstrated the poor mixing in the reactor, and the low oxygen transfer rate that still prevailed despite sparging with gas. Dissolved oxygen profiles were created with pure oxygen sparged into the reactor. Even at this high concentration of oxygen and high flow rate, complete utilization of oxygen by the *Staph. epidermidis* cells occurs. It is assumed that these low levels of dissolved oxygen are likely to create an oxygen-limited environment, which alters the susceptibility of biofilms to antibiotic attack (Borriello *et al.* 2004). The lack of axial flow in the system may be a reason for the profiles that tend towards 0 mg l^{-1} . The possibility of introducing a second sparger and further gas to increase the levels of dissolved oxygen in the media was hampered by the foaming that occurred when gas flow rates $>0.5 \text{ l min}^{-1}$ were used. This may be

related to the size of the bubbles of gas sparged into the reactor. Smaller bubbles would enable greater oxygen transfer into the liquid, but overall it must be concluded that the mRDR significantly increased the amount of oxygen that could be transferred into the liquid when compared with the cRDR.

Although the 0 and 21% sparged oxygen profiles look identical from 7 h onwards, it is important to note that there is a difference in the two conditions; cultures sparged with 0% oxygen are forced to grow anaerobically, whereas cultures sparged with air consume the amount of oxygen at the rate supplied. The sparged oxygen concentration results in the difference in biofilm accumulation, where a statistical difference was previously noted between the 0 and 21% sparged oxygen concentrations (Cotter *et al.* 2009).

The majority of staphylococcal strains that form biofilm are facultative anaerobes; though, some strict anaerobes have been isolated (Rowlinson *et al.* 2006). Oxygen conditions vary *in vivo*, and differences in the response of staphylococci are noted, particularly in regard to their production of extracellular polysaccharides (Mckenney *et al.* 2000). Expression of PIA, a key extracellular polysaccharide produced by Staphylococci, increases under anaerobic conditions (Cramton *et al.* 2001), as oxygen alters the physiology of the cells. These factors must be considered when attempting to understand the utilization of available oxygen by planktonic cells and the switch to a biofilm phenotype in aerobic and anaerobic conditions.

Analysis of biofilm thickness

The influence of oxygen on biofilm formation, quantified by adherence of viable cells, translated well in thickness measurements of samples that were cryosectioned and viewed at 40 \times . Data from the slices showed a statistical difference between the 0 and 21% sparged oxygen biofilms, the same conditions that showed a difference in the CFU counts. The thickness data tested may not be as statistically convincingly as the corresponding CFU data. The resultant images are of such a small area that comparison in thickness measurements, which are taken directly from these images, may not accurately reflect the differences in biofilm coverage on the coupon. However, from the small area imaged the difference in viable cells does translate to a thickness difference.

Gradients of respiratory activity in the biofilm samples were not visible using CTC and DAPI in combination; however, these stains have previously been proven to highlight the gradients where present in biofilm systems (Heffernan *et al.* 2009). There is no fundamental reason why the RDR could not be used to view gradients in oxygen or nutrients in a biofilm, if present, provided the cor-

rect staining methods are employed. This could result in the RDR coupon system being a convenient way of examining biofilms for gradients prior to removing the entire biofilm for mRNA extraction. It has been shown that zones of DNA synthetic activity and oxygen respiration correspond in *Staph. epidermidis* biofilms (Rani *et al.* 2007), which could lead to the difference in mRNA expression levels depending on where in the biofilm the cells originated from. If no gradients are detected, such as in this case where homogeneity in respiratory activity is present, it eliminates the weakness of using whole biofilm populations to execute mRNA expression profiling.

Antibiotic susceptibility

Biofilms of *Staph. epidermidis* cultivated on mRDR coupons were analysed for the susceptibility to rifampicin. At the highest concentration of rifampicin tested (2 $\mu\text{g ml}^{-1}$), a large quantity of biofilm remained, and the antibiotic failed to eradicate c. 84% of the viable cells in both the 0 and 21% sparged oxygen biofilm samples. The statistically insignificant biofilm susceptibility ($P = 0.18$ by performed using a two-way analysis of variance (ANOVA)) under different oxygen concentrations measured here is consistent with the data of Nordern and Shaffer (1983), who reported that minimum bactericidal concentrations of rifampicin challenging *Staph. aureus* were identical under aerobic and anaerobic conditions. Zheng and Stewart (2004) found similar results, but both studies concentrated on the atmospheric oxygen conditions that the rifampicin treatment was performed in. This is the first time biofilm cultured in conditions where the sparged oxygen into the media is controlled. The biofilm is cultivated in a defined sparged oxygen concentration, and rifampicin treatment also takes place in the same oxygen conditions.

The method of antibiotic treatment of biofilm involved washing the antibiotic from the coupon prior to slicing or quantification of viable cells. For the subsequent thickness measurements taken from cryosectioned slices, it is hypothesized that this essential washing step may have removed the dead cells on the top layer of the biofilm, which may account for the erratic nature of the thickness measurements that show no statistical differences.

The potential use of additional fluorescent markers prior to cryoslicing, or susceptibility testing with different or combinations of antibiotics, enables further analysis to be performed using biofilm cultivated on RDR coupons.

Conclusion

The commercial RDR is used to grow monoculture biofilm and has been modified to allow greater control of

oxygen levels in the nutrient media. The addition of a sparger, which allowed greater mass transfer of oxygen into the media, also enforced a baffle effect on the system which reduced the vortex caused by the rotation of the disk. Results from the mRDR showed that oxygen concentration plays an important role in the quantity of *Staph. epidermidis* biofilm forming on the removable coupons.

The versatility of the RDR and the modifications made to it in this work identify it as a reactor with potential for cultivating pure culture biofilm in defined, controlled conditions. The range of *ex situ* analysis described herein, including biofilm and planktonic CFUs, with staining and cryoslicing of biofilm, is performed on biofilm cultivated under defined oxygen concentrations, indicating the potential for the RDR to become an important, commonly used biofilm reactor.

Limitations in the mRDR were observed when an increase in the gas flow rate above 0.5 l min⁻¹ resulted in foaming of the media, which could wet the inlet or outlet filter and compromise the aseptic environment. Increased agitation speed also has an effect on foaming, and the stability of the disk became compromised above 300 rev min⁻¹.

Antibiotic susceptibility of biofilm cultivated in different oxygen concentrations was also examined, with different concentrations of rifampicin. The concentration of oxygen sparged into the growth media for the biofilm made no statistical difference to the effectiveness of the antibiotic, concurring with previous results (Zheng *et al.* 2004).

Modifications to the reactor greatly increase the oxygen transfer rate, and with evidence of the influence that oxygen has on *Staph. epidermidis* biofilm formation shows an important improvement in the development of the rotating disk system. Although higher oxygen transfer rates are desirable, the mRDR facilitates a large range of analysis that can be performed with biofilm grown on its removable coupons and is a potential solution for growing monoculture biofilm under defined oxygen concentrations and performing a multitude of quantification and image-based analysis.

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