<table>
<thead>
<tr>
<th>Title</th>
<th>Rapid depletion of dissolved oxygen in 96 well microtitre plate Staphylococcus epidermidis biofilm assays promotes biofilm development and is influenced by inoculum cell concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Cotter, John J.; O'Gara, James P.; Casey, Eoin</td>
</tr>
<tr>
<td>Publication date</td>
<td>2009-08-01</td>
</tr>
<tr>
<td>Publication information</td>
<td>Biotechnology and Bioengineering, 103 (5): 1042-1047</td>
</tr>
<tr>
<td>Publisher</td>
<td>Wiley</td>
</tr>
<tr>
<td>Link to online version</td>
<td><a href="http://dx.doi.org/10.1002/bit.22335">http://dx.doi.org/10.1002/bit.22335</a></td>
</tr>
<tr>
<td>Item record/more information</td>
<td><a href="http://hdl.handle.net/10197/2729">http://hdl.handle.net/10197/2729</a></td>
</tr>
<tr>
<td>Publisher's statement</td>
<td>This is the authors' version of the following article: &quot;Rapid depletion of dissolved oxygen in 96-well microtiter plate Staphylococcus epidermidis biofilm assays promotes biofilm development and is influenced by inoculum cell concentration&quot; published in Biotechnology and Bioengineering 103:5 pp 1042–1047. It is available in its final form at <a href="http://dx.doi.org/10.1002/bit.22335">http://dx.doi.org/10.1002/bit.22335</a></td>
</tr>
<tr>
<td>Publisher's version (DOI)</td>
<td><a href="http://dx.doi.org/10.1002/bit.22335">http://dx.doi.org/10.1002/bit.22335</a></td>
</tr>
</tbody>
</table>
Rapid depletion of dissolved oxygen in 96 well microtitre plate *Staphylococcus epidermidis* biofilm assays promotes biofilm development and is influenced by inoculum cell concentration

John J. Cotter\textsuperscript{1}, James P. O’Gara\textsuperscript{2} and Eoin Casey\textsuperscript{1}*.

\textsuperscript{1}UCD School of Chemical and Bioprocess Engineering, Centre for Synthesis and Chemical Biology, and \textsuperscript{2}UCD School of Biomolecular and Biomedical Science and UCD Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland.

*Corresponding Author: Eoin Casey.

Email: eoin.casey@ucd.ie

Telephone: +353 1 7161877

Fax: +353 1 7161177
Abstract

Biofilm-related research using 96 well microtitre plates involves static incubation of plates indiscriminate of environmental conditions, making oxygen availability an important variable which has not been considered to date. By directly measuring dissolved oxygen concentration over time we report here that dissolved oxygen is rapidly consumed in *Staphylococcus epidermidis* biofilm cultures grown in 96 well plates irrespective of the oxygen concentration in the gaseous environment in which the plates are incubated. These data indicate that depletion of dissolved oxygen during growth of bacterial biofilm cultures in 96 well plates may significantly influence biofilm production. Furthermore higher inoculum cell concentrations are associated with more rapid consumption of dissolved oxygen and higher levels of *S. epidermidis* biofilm production. Our data reveal that oxygen depletion during bacterial growth in 96 well plates may significantly influence biofilm production and should be considered in the interpretation of experimental data using this biofilm model.

Introduction

96 well plates have mainly been applied to routine laboratory assays and high throughput drug discovery, many in automated processes (Sandberg *et al.*, 2008). Originally used as batch “mini-bioreactors” to maintain clonal libraries of facultative anaerobes such as *Escherichia coli* and yeasts, some recent studies have examined mixing and mass transfer of oxygen into the liquid media (Zhang *et al.*, 2008). Variations in shaking the plates and adjustments to improve mass transfer have been performed (Micheletti *et al.*, 2006), but plates are still often used statically in microbiological experiments, particularly for
biofilm assays. In the case of aerobic fermentations using fast growing cultures, oxygen quickly becomes rate limiting, because the solubility of oxygen in water/growth medium is low (Shin et al., 1996).

Communities of bacteria that adhere to a surface and grow in a matrix-enclosed structure are known as biofilms (Characklis and Cooksey, 1983). Macro scale biofilm systems, such as glass capillary flow cells (Stoodley et al., 2001), the rotating disk reactor (Zelver et al., 1999) and the CDC reactor (Goeres et al., 2005), are commonly used to study biofilm characteristics, such as structure and susceptibility to antibiotics (Stewart and Costerton, 2001).

Polystyrene 96-well plates have become one of the most popular methods for micro scale biofilm investigations (Christensen et al., 1985) and are commonly used in biofilm genetic studies (Davey and O’Toole, 2000) to analyse differences in the quantity of biofilm formation in engineered mutants. A downside of this technique is the considerable experiment to experiment variation in biofilm production by individual bacterial strains. Nevertheless the technique is useful in determining whether bacterial strains are capable of forming biofilm (Peeters et al., 2008). Specific modifications have been made to the basic 96 well plate method (Christensen et al., 1985) by different groups, particularly in terms of the inoculum cell concentration and culture volumes. Table 1 indicates the range of inoculum cell concentrations and culture volumes used in Staphylococcus epidermidis 96 well plate biofilm assays. Biofilm experiments using 96 well plates are typically incubated at the optimal growth temperature without shaking and without consideration to the oxygen demand of the cells.
In vitro studies of staphylococcal biofilm development have revealed that anaerobic conditions promote production of the icaADBC operon encoded polysaccharide intercellular adhesin (PIA) (Heilmann et al., 1996), an important component of the staphylococcal biofilm matrix, in both S. aureus and S. epidermidis (O’Gara, 2007). Oxygen limitation may therefore influence biofilm thickness. It can reasonably be assumed that in thicker biofilms, nutrient limitation is more likely at the substratum-biofilm interface, which in turn will influence biofilm physiology (Rani et al., 2007). Slow-growing cells under nutrient-limited conditions have been shown to be more resistant to antibiotics (Xu et al., 2000). Increased biofilm thickness also results in more locally anoxic regions or “pockets” forming within the deeper regions (Rani et al., 2007).

The objective of this work was to directly measure levels of dissolved oxygen available to S. epidermidis 1457 cells forming biofilm in 96 well plates, in order to determine if oxygen limitation influences the biofilm phenotype in these temperature and oxygen-controlled conditions.

Materials and Methods

Bacterial strain 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 brain heart infusion (BHI, Oxoid) broth overnight at 37°C.

Biofilm quantification in 96 well plates. Overnight cultures were adjusted to $A_{660}=1.0$ using sterile BHI to prepare a standard inoculum for biofilm assays. This standard inoculum equated to log 7.84 ± 0.01 colony forming units (CFU) ml$^{-1}$. Experiments were also undertaken using lower and higher inoculum cell concentrations. The “low” inoculum consisted of log 6.43 ± 0.02 CFU ml$^{-1}$ and the “high” inoculum represented log
8.97 ± 0.01 CFU ml⁻¹. Both were created by dilution of the overnight culture with sterile BHI, adjusted to different densities. 1 in 100 μl dilutions of the low, standard and high inoculums were added to each well in the presence of the filtered gas in which the biofilm was to be cultured. 100 μl was used in all wells for all reported experiments. Lids were placed on the plates before incubation at 37°C, in a 10 l sterile sealed vessel, with a constant stream of filtered gas passed through the headspace. The biofilm plates were incubated in an atmosphere of 100% oxygen, or 21% oxygen / 79% nitrogen. All concentrations were verified off line prior to operation using a gas analyser (Servomex 1400 gas analyzer, Sussex, U.K.). Evaporation from the wells caused a negligible difference in liquid volume in the different oxygen environments (data not shown).

Quantification of planktonic cells from 96-well plates was performed as described previously, with 100 μl of the culture being mixed with 900 μl sterile Ringers solution and serially diluted before being plated on BHI agar plates. To quantify the biofilm adhering to the wells, the liquid culture was removed and the wells washed rigorously three times with sterile Ringers solution to remove all planktonic cells. After the third wash, 100 μl of Ringers solution were added to the wells and the plate was sonicated for 1 min to separate the biofilm from the base of the plate. The ultrasonic waves lifted the biofilm from the plate into the Ringers, and this solution was further vortexed for 2 min to ensure dispersal of any cell aggregates prior to performing serial dilutions and total viable counts as described above.

**Dissolved oxygen measurements of planktonic cells.** For direct dissolved oxygen readings, 96 well plate lids were pierced with a sterile 18G needle under aseptic
conditions and covered with UV sterilized parafilm. This enabled insertion of the needle-type oxygen microsensor (PreSens GmbH, Regensburg, Germany) into the centre of individual wells of a 96 well plate, 2mm from the top and bottom of the liquid, without removing the lid. From incubator to final measurement, the entire process took less than 2 min, which included the time allowed for the probe to stabilise and give a steady reading. Readings were the average of four measurements and were recorded every second. The dissolved oxygen in three wells from two plates was measured and the mean and standard error are presented.

**Statistical analysis.** Statistical analysis of dissolved oxygen, biofilm accumulation and total cell growth was performed by a two way analysis of variance (ANOVA).

**Results**

**Dissolved oxygen is rapidly consumed by bacterial cultures in 96 well plate biofilm assays.** Using a narrow tip oxygen sensor probe as part of a novel application, direct measurements of dissolved oxygen within an inoculated well of a 96 well plate were recorded. In Fig. 1A, *S. epidermidis* 1457 was cultured in a headspace atmosphere of air (21% oxygen). Complete depletion of dissolved oxygen was measured after 6 h. The same experiment performed in an atmosphere of 100% oxygen (Fig. 1B), also revealed that the dissolved oxygen concentration fell below the detectable level after 6 h but ultimately recovered to previously observed levels, and remained stable at ~ 7 mg L\(^{-1}\) after 24 h. As previously observed for this strain, less biofilm formed when oxygen was abundantly available (Cotter *et al.*, 2009).

**Oxygen consumption promotes biofilm growth by *S. epidermidis* 1457 in 96 well microtitre plates.** Profiles of dissolved oxygen concentrations and numbers of
planktonic cells at different oxygen concentrations are shown in Fig. 2. The total biofilm formation of *S. epidermidis* 1457 in each well is shown as “Total biofilm CFU” in this figure. The initial dissolved oxygen concentration is that of autoclaved BHI media and is the same for all profiles. In an atmosphere of 0% or 21% oxygen, when dissolved oxygen was consumed it remained below detection for the remainder of the experiment (up to 24 h), though only the initial hours are shown in Fig. 2. A profile of dissolved oxygen concentrations in 96 well plates incubated in an atmosphere of 100% oxygen conditions is shown in Fig. 1B. The ratios of planktonic to biofilm cell counts in 96 well plates incubated in an atmosphere of 0%, 21% or 100% oxygen are shown in Fig. 2D. Predictably, at higher atmospheric concentrations of oxygen, the dissolved oxygen concentrations in 96 well plates take longer to be depleted. Biofilm cell numbers were statistically significantly higher under anaerobic conditions compared to 21% and 100% oxygen conditions (p<0.05). Indeed, the ratio of planktonic to biofilm cell numbers revealed that overall in the 96 well plate cultures a higher fraction of the biomass exists as biofilm under anaerobic compared to aerobic conditions.

**Inoculum cell concentrations influence oxygen consumption and biofilm formation.** Three different initial cell concentrations (low, standard and high inocula) of *S. epidermidis* 1457 were used to inoculate 96 well plate cultures in an atmosphere of 100% oxygen. Fig. 3 shows dissolved oxygen concentrations, biofilm cell numbers, total bacterial cell numbers and the ratio of planktonic to biofilm cell numbers under these conditions. The ratio of planktonic to biofilm cell numbers reaches $1.4 \pm 0.15$ after 4 h irrespective of the inoculum cell concentration. The oxygen demand in the first few hours of growth depends strongly on the cell concentration of the inoculum, as the
different biomass quantities are associated with different dissolved oxygen concentrations. The onset of oxygen limitation occurs at approximately 1, 3 and 6 h for the high, standard and low inoculum cell concentrations respectively. Statistical differences are observed from hours 1-4 in graphs A-C between the high inoculum and the other inocula (p<0.05). A difference is noted between the total bacterial growth in the standard and low inocula (p<0.05). The development of biofilm following inoculation with high, standard and low cell concentrations, arbitrarily defined as > $10^4$ CFU cm$^{-2}$, occurs after 1 h, 1 h and 3 h, respectively (Fig. 3B), apparently reflecting the rate at which dissolved oxygen is consumed within these cultures.

Discussion

In this study we profiled dissolved oxygen concentrations during growth of bacterial biofilms in 96 well microtitre plates. Despite the pleiotrophic impact of oxygen on bacterial cell physiology, biofilm-related research using 96 well plates has thus far failed to consider the role of oxygen availability and demand. Previous reports identified a maximum oxygen transfer level of 0.03 - 0.035 mol/ L / h in planktonic cultures grown a standard shaken 96-well plate (Duetz et al., 2000). This may not be sufficient for growth of actively metabolizing aerobic microorganisms, and thus becomes an issue for use of the plates as “mini-bioreactors” (Samorski et al., 2005). Oxygen transfer limitation is also of importance for biofilm growth, where spatial stratification of oxygen can lead to localised anoxic regions in the biofilm adjacent to the substratum (Rani et al., 2007). We have previously shown that anaerobic conditions activated transcription of the icaADBC operon and biofilm development in  $S$ epidermidis by increasing the activity of the alternative sigma factor $\sigma^B$, which in turn down-regulates expression of the icaR
repressor (Cotter et al., 2009). The results presented in this manuscript correlate well with our previous data (Cotter et al., 2009), as the quantity of biofilm formation was higher when the 96 well plates were incubated in an anaerobic environment. Our previous study also revealed that the *S. epidermidis rsbU* transposon mutant M15 (Mack et al., 2000) was incapable of biofilm production in an anaerobic environment, but did form biofilm at high oxygen concentrations (Cotter et al., 2009). Previous experiments with this *S. epidermidis* σB mutant were performed in 96 well plates (Knobloch et al., 2001). The data presented in this study reveal that dissolved oxygen concentrations are low during bacterial growth in 96 well plate biofilm assays and therefore serve to highlight the importance of oxygen in this biofilm experimental model and indeed an important limitation of this approach.

Our data also reveal that inoculum cell concentrations influence biofilm development. A wide range of inoculum cell concentrations and culture volumes, both of which have implications for oxygen utilisation, have been used for cultivation of *S. epidermidis* biofilms in 96 well plates (Table 1). Interestingly, Sandberg et al. (2008) grew *S. aureus* in an aerobic environment at 200 rpm, revealing an inoculum cell concentration of $\sim 10^6$ CFU ml$^{-1}$ promoted more biofilm formation after 18 h in 96 well plates than a higher inoculum cell concentration of $\sim 10^8$ CFU ml$^{-1}$. Results in fig. 3, which were performed statically in a 100% oxygen environment, may differ to those conclusions due to the availability of oxygen. We conclude that 96 well plate inoculum cell concentrations influence the time at which the onset of oxygen limitation occurs, concomitantly influencing biofilm formation. This study reveals that dissolved oxygen is rapidly consumed in staphylococcal biofilm cultures grown in 96 well microtitre plates, resulting
in persistent or transient anaerobic conditions depending on the prevailing atmospheric oxygen concentrations. Given the importance of oxygen availability for bacterial physiology and biofilm formation, these data highlight a significant limitation of this technique, and it is suggested that both inoculum cell concentrations and culture volumes should be standardized in 96 well plate biofilm assays. It is also worth noting that, although not an objective of the present study, the availability of oxygen microsensors has created the potential to develop a methodology for the precise control dissolved oxygen levels in the liquid phase of microwells.

**Acknowledgements**

This research was funded by Science Foundation Ireland (SFI) grant 04/BRG/E0072. We thank Eoin Syron and Sharon Davin for technical support. Strain *S. epidermidis* 1457 was a kind gift from Dietrich Mack, University of Swansea, Wales, U.K.


Figure Legends

1. Biofilm formation (●), planktonic growth (■) and dissolved oxygen (▲) in liquid cultures of *S. epidermidis* 1457, grown at 37°C for 24 h in plates incubated in air (21% oxygen) (A) or an atmosphere of 100% oxygen (B).

2. **Influence of atmospheric oxygen concentrations on *S. epidermidis* planktonic and biofilm growth in the early hours of a 96 well biofilm assay.** *S. epidermidis* 1457 grown in 96-well plates in an atmosphere of 0% oxygen (●), 21% oxygen (○) and 100% oxygen (▼) for 6h. (A) Dissolved oxygen concentrations, (B) total biofilm formation (CFU), (C) Total bacterial cell growth (planktonic and biofilm) (CFU), and (D) ratio of planktonic to biofilm CFU counts, indicating the rate of biofilm conversion for the different oxygen concentrations. Error bars are the standard error of three wells from two independent plates.

3. **Influence of inoculum cell concentration on *S. epidermidis* biofilm production in the early hours of a 96 well plate biofilm assay.** Low (●), standard (○) and high (▼) inoculums of *S. epidermidis* 1457 grown in 96-well plates, incubated in an atmosphere of 100% oxygen. (A) Dissolved oxygen, (B) total biofilm formation (CFU), (C) Total growth of biofilm + planktonic CFU, and (D) ratio of planktonic to biofilm CFU counts.
Fig. 1

A

Time (h)

Biofilm Log CFU cm\(^{-2}\) ± standard error

Planktonic Log CFU ml\(^{-1}\) ± standard error

Dissolved Oxygen (mg L\(^{-1}\))

B

Time (h)

Biofilm Log CFU cm\(^{-2}\) ± standard error

Planktonic Log CFU ml\(^{-1}\) ± standard error

Dissolved Oxygen (mg L\(^{-1}\))
Fig. 2
Fig. 3

A

Dissolved Oxygen (mg L\(^{-1}\))

Time (h)

B

Log CFU cm\(^{-2}\) ± standard error

Time (h)

C

Total Log CFU ± standard error

Time (h)

D

Planktonic CFU / Biofilm CFU

Time (h)
Table 1. A selection of inoculum cell concentrations and culture volumes of *Staphylococcus epidermidis* strains used in 96 well plate biofilm assays.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Inoculum</th>
<th>Culture Volume</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 35984</td>
<td>$4 \times 10^7$ - $1 \times 10^9$ CFU ml$^{-1}$</td>
<td>100 μl</td>
<td>Dunne <em>et al</em>., 1991</td>
</tr>
<tr>
<td>CSF 41498</td>
<td>Undiluted overnight culture</td>
<td>100 μl</td>
<td>Conlon <em>et al</em>., 2002</td>
</tr>
<tr>
<td>ATCC 35984</td>
<td>10% of overnight culture</td>
<td>200 μl</td>
<td>Peeters <em>et al</em>., 2008</td>
</tr>
<tr>
<td>ATCC 35983, 35984, 35981, 35982</td>
<td>dilution of overnight culture</td>
<td>200 μl</td>
<td>Christensen <em>et al</em>., 1985</td>
</tr>
<tr>
<td>ATCC 55113, SE1175</td>
<td>1: 50 dilution of overnight culture</td>
<td>200 μl</td>
<td>Wu <em>et al</em>., 2003</td>
</tr>
<tr>
<td>1457, NJ9709</td>
<td>$10^3$ - $10^5$ CFU ml$^{-1}$</td>
<td>200 μl</td>
<td>Izano <em>et al</em>., 2008</td>
</tr>
<tr>
<td>1457, ATCC 35984</td>
<td>1:200 dilution of overnight culture</td>
<td>200 μl</td>
<td>Heilmann <em>et al</em>., 1996</td>
</tr>
</tbody>
</table>