The effect of agitation on bacterial growth, biofilm production and release from matrices

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Abstract

The effects of agitation provided by a novel shaker on the rate of bacterial growth was investigated using varying geometric patterns, speeds and stroke distances. These were compared to conventional shakers and shaker settings. Growth rates and generation times were calculated from growth curves for Escherichia coli, Pseudomonas aeruginosa *and* Staphylococcus aureus. *Under the different settings, the generation times of these bacteria were not significantly shorter than when grown in conventional shakers.*

The formation of biofilm on prosthetic devices is a cause of many infections in arthroplasty patients. The effects of agitation on the formation of biofilm was also investigated for its possible application to prosthetic devices. S. epidermidis *and* P. aeruginosa *were grown under dynamic and static conditions. Growth of bacteria under these conditions yielded the same amount of biofilm in both static and shaking culture. Both these findings are contrary reports in the literature.*

1.0 Introduction

1.1 Microbial growth

1.1.1 Phases of growth

In a closed culture, bacteria show a distinct pattern of growth which can be depicted as a bacterial growth curve when bacterial numbers are plotted on a logarithmic graph over time (Figure 1.1). In this curve, there are four phases of growth. The lag phase occurs after bacteria have been introduced to a new environment, and growth does not occur for a period as the bacteria adjust. In the log phase, bacteria exhibit the most active binary fission and cellular reproduction. The generation time is constant, leading to logarithmic growth. Bacteria are particularly vulnerable to adverse conditions during this phase. Stationary phase occurs when limiting factors slow or stop bacterial growth, resulting in numbers of bacteria remaining static. Finally, the build up of harmful metabolites and the exhaustion of nutrients inevitably occurs, leading to the death of cells exceeding the number of new cells formed, and a steady decline in bacterial numbers (Tortora *et al.,* 2004).

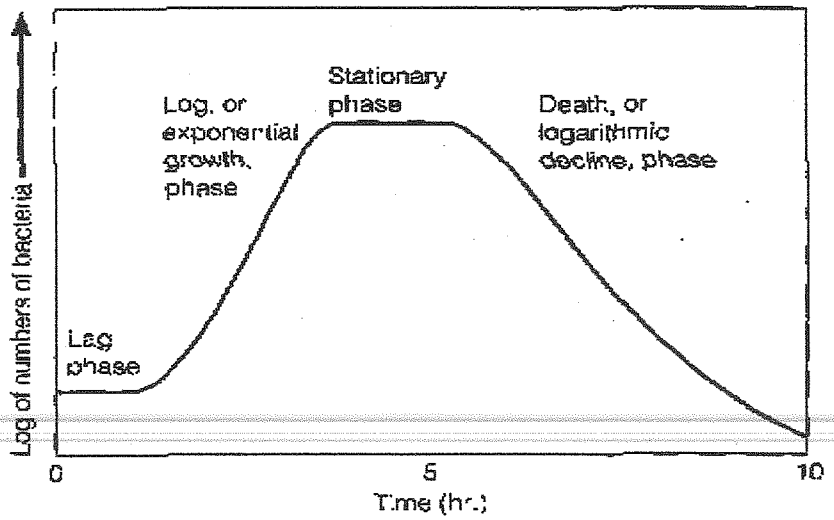

Figure 1.1 – A bacterial growth curve showing the four phases of growth (Tortora *et al.* 2004).

1.1.2 Growth manipulation

In clinical laboratories, enhancement of bacterial numbers also occurs by growing bacteria at optimal conditions (Buchs, 2001). In the case of facultative anaerobes (bacteria which can grow in oxygenated or anaerobic conditions) and obligate aerobes (bacteria requiring oxygen for growth), increased aeration is a common practise. This is usually achieved by increasing the amount of oxygen present, either by pumping air or oxygen directly into cultures (known as gas sparging), or by shaking (Buchs, 2001; Reyes *et al.*, 2003; Puskeiler *et al.*, 2004; Doig *et al.*, 2005a; Doig *et al.*, 2005b; Kensy *et al.*, 2005).

1.2 Conventional shakers and the Planetary Electric Motor (PEM)

Conventional shaker designs used in clinical laboratories are the orbital and reciprocating shakers. However, these have many limitations, including having only one geometric pattern, a small range of shaking speeds and only one shaking size or diameter of movement. The Planetary Electric Motor (PEM) developed by VibraQ Corporation allows for a new shaker design, resulting in possible improvements in culture aeration in various applications. The PEM shaker can perform complex geometric patterns, and speed and magnitude of movement is also adjustable. The PEM shaker has already been used to increase the shelf-life of blood platelets by the Australian Red Cross and to treat lymphoedema (B Hobson, VibraQ, personal communication).

1.3 Biofilms

Biofilms can be defined as "a microbially derived sessile community characterised by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription" (Donlan and Costerton, 1989). The formation of biofilms increases bacterial survival though protection from the outside environment (such as UV radiation) and resistance to antimicrobial agents, heat, cold, and bacteriophages (Carpentier and Cerf, 1993; Garcia-Saenz *et al.,* 2002). In prosthetic device infections, biofilm formation is part of the infection process. Infection usually results in prosthetic excision.

The initial stage of biofilm formation is cell attachment. Some studies have shown that when microbes are grown under dynamic conditions, biofilm formation is reduced (Stepanovic *et al.,* 2001; Bechet and Blondeau, 2003; Stepanovic *et al.,* 2003).

In this study, the effects of shaking on bacterial growth were investigated. In addition, the effect of shaking on the attachment of bacterial cells to surfaces was explored.

2.0 Methods and Materials

2.1 Growth curves

2.1.1 Experimental design

Eight hour growth curves were constructed for *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. For comparison, eight growth curves were constructed with shaking by an orbital shaker and seven trials with a reciprocating shaker. Initial trials with the PEM shaker were conducted using 21 patterns where each trial had differing geometric patterns, magnitudes and speeds. Nine of these trials that showed promising results were repeated. From these, three sets of parameters were chosen to be repeated using a modified method. Flasks which had been initially enclosed with non-absorbent cotton wool were sealed with surgical masks in this modified method.

In all growth curve experiments, optical density readings at 600nm (OD_{600}) and viable counts were made every hour.

2.1.2 Data analysis

Viable counts were used to determine the growth rate and generation time of each bacterial species. A nonparametric statistical test (Mann-Whitney *U* Test) was used to determine whether there was a significant difference in generation times between the PEM shaker and the orbital and reciprocating shakers.

2.2 Biofilm formation

Biofilm forming strains of *S. epidermidis* and *P. aeruginosa* were inoculated into 96-well Nunc© plastic microtiter plates. A non-biofilm forming strain of *E. coli* was used as a control. These were incubated at 37°C either statically or with shaking, and readings taken at 4, 6 and 8 h.

Readings were taken by washing free-floating cells from each well and fixing the remaining attached cells (the biofilm) with methanol. The biofilm was stained and excess stain rinsed from the wells. The stain was then resolubilised using 33% (v/v) glacial acetic acid, and the OD_{570} taken.

This process was repeated two times for two patterns on the PEM shaker.

3.0 Results and discussion

3.1 Growth curves

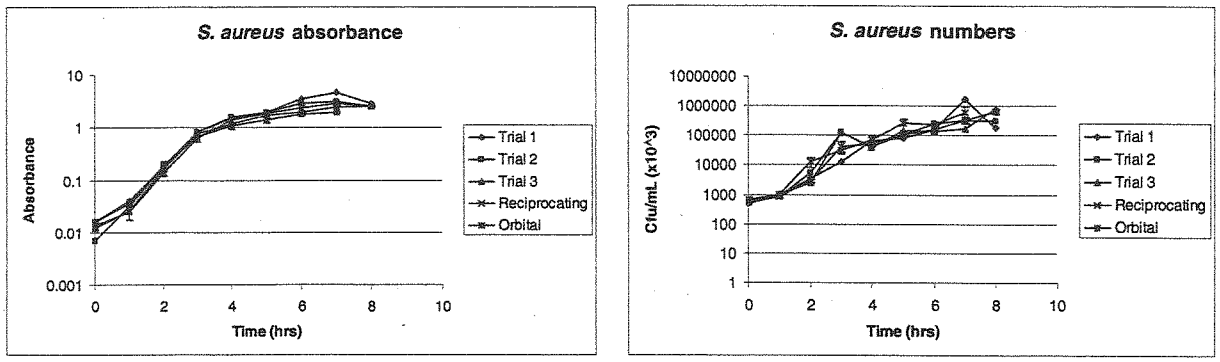

Figure 3.1.1 – *S. aureus* absorbance (left) and number of viable (living) cells when grown using geometric pattern X at three different settings.

After initial experiments the PEM shaker there was found to be no statistically significant differences in yielded generation times from generation times of *E. coli*, *S. aureus*, and *P. aeruginosa* grown in the orbital and reciprocating shakers. Representative growth curves for *S. aureus*, using OD_{600} and viable counts, are shown in Figure 3.1.1. The PEM shaker had shown many promising outcomes but when subjected to the adopted statical method they did not reveal significance. This was not expected, as previous experiments had shown that if there is enough oxygenation occurring, bacteria will grow quicker at higher shaking speeds, especially when smaller volumes of sample are being used (McDaniel and Bailey, 1969; Delgado *et al.*, 1989).

The lack of increased growth could have been due to a number of reasons. First, to show statistically significant differences in growth curves requires repeated experimental tests. Potentially promising trial patterns were chosen based on high absorbance measurements, high numbers of organisms grown and/or quick generation times. These nine trials are to be repeated once and the results again evaluated.

Another possible reason for the lack of increased growth in these trials is that, in all cases (orbital, reciprocating and PEM shakers), flasks were sealed with a cotton wool stopper to prevent contamination. This has been shown to restrict the amount of aeration of the culture (Gaden, 1962; McDaniel and Bailey, 1969; Gupta and Rao, 2003). All organisms may have been starved of oxygen, restricting their growth. In order to test this, after repeat trials were performed, three PEM trials were done with a modification that allowed more oxygenation. This involved replacing the cotton wool stopper with a surgical mask cover. Similar modifications were made with the orbital and reciprocating shakers. Small volumes of media were also used for all growth curves (50mL in 250mL conical flasks), which contained more air in the head of the flask.

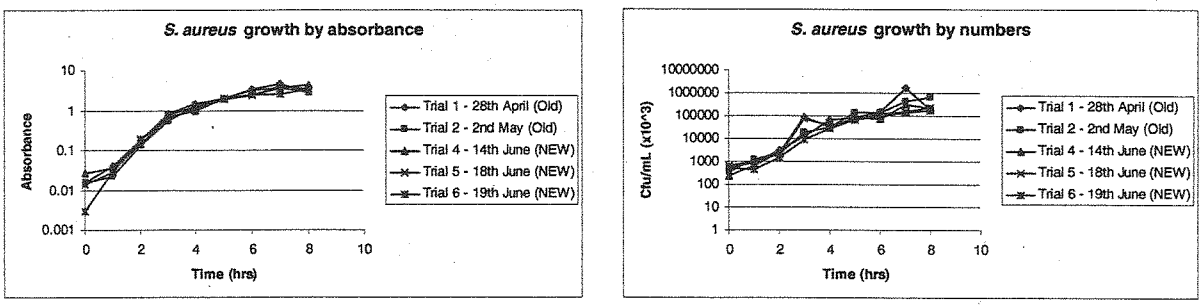

Figure 3.1.2 – *S. aureus* absorbance and viable counts when grown under Pattern X, Trial 1 conditions (from Figure 3.1.1). In the old method, flasks were closed with non-absorbent cotton wool. In the new method, flasks are covered with surgical masks.

Representative growth curves for *S. aureus,* using OD_{600} and viable counts, following the modification are shown in Figure 3.1.2. The increased oxygenation in the PEM shaker did not yield generation times which differed significantly from generation times of *E. coli*, *S. aureus*, and *P. aeruginosa* grown in the orbital and reciprocating shakers. There are two possible reasons that this would occur. Again, it is likely that not enough trials were conducted to give an accurate statistical analysis by the Mann-Whitney *U* test. Second, there may be sufficient oxygen available for the bacteria to reach maximum growth in the first method (using a cotton-wool plug); therefore, modifications allowing increased oxygenation would not make a difference to growth rate.

3.2 Biofilm formation

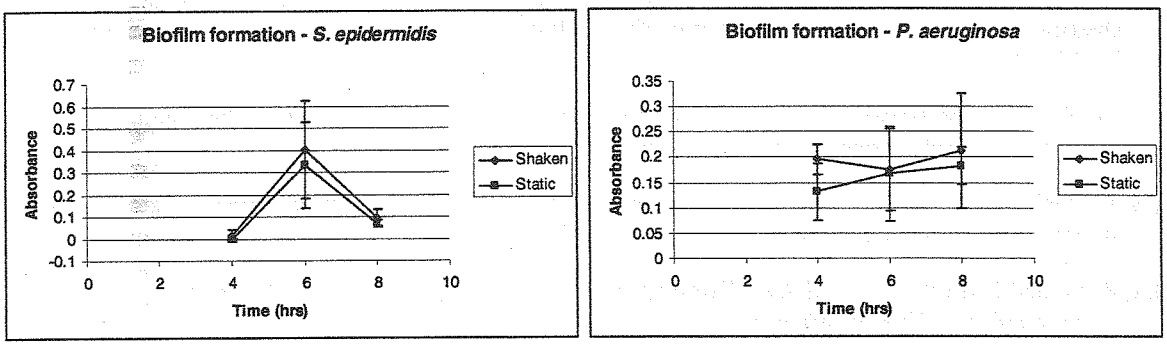

Figure 3.2.1 – *S. epidermidis* (left) and *P. aeruginosa* (right) absorbance when forming biofilm under static conditions and dynamic conditions (using setting Y). Standard deviation for two tests is shown.

In the two settings under which biofilm formation was tested, both appeared to show no difference between the amount of biofilm formed under static and dynamic conditions. Statistical analysis was not performed.

4.0 Conclusion

In investigating the effects of agitation on bacterial growth, the growth rate and generation times of *E. coli*, *S. aureus* and *P. aeruginosa* remained similar regardless of the geometric pattern, magnitude or speed at which they were shaken. This is contrary to many papers which have investigated these factors in the past (Gaden, 1962; McDaniel and Bailey, 1969; Buchs, 2001; Gupta and Rao, 2003). More studies into this area need to be undertaken in order to gain further knowledge of the PEM shaker.

Also, dynamic factors made no difference to the amount of biofilm formed. This is in contrast to the fingins of some (Bechet and Blondeau, 2003), particularly literature focussed on agitating staphylococci (Stepanovic *et al.,* 2001) where *S. epidermidis* was found to have reduced biofilm formation under conditions similar to those in the present study. Because only a few trials have been completed, more are needed before an accurate statistical analysis can be undertaken using different agitation settings in order to make valid conclusions regarding the influence of agitation on biofilm formation.

5.0 Acknowledgements

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