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Improving phage titre through examining point of infection

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Abstract

Bacteriophages are viruses that cause the lysis of bacteria. They have recently been used to combat antimicrobial resistant infections as an alternative therapy to antibiotics. Their production and propagation, however, remains understudied and will be key to obtaining titres required for future clinical studies and research. Previous work suggests that temperature of infection significantly influences the production process and output yield of phage, with a reduction in temperature from 37ºC to 28ºC resulting in significant increases in productivity for multiple host-phage systems. The current study aimed to build upon this previous work by examining different temperature conditions at the point of infection to determine the effect on harvest phage titre improvements. Investigations were conducted at different culture scales ranging from 20mL shake flasks to 3L stirred tank bioreactor cultures to investigate process differences when scaling from laboratory bench-scale to initial industrial scale fermentations. Additionally, the kinetics of phage infection were investigated. In small scale cultures, the greatest phage bursts and harvest titres were generated by maintaining cultures under static 28 o C incubation during infection compared to agitation and temperature reduction from 37 o C. Investigating the dynamics around the point of infection will help to inform scalable processes for manufacture of phage for a variety of applications ranging from direct therapeutic application to self-assembling bio-templates for nanostructure synthesis.

Core Findings

- Reducing the temperature of infection from 37^{o}C to 28^{o}C using a static incubator improves titre
- An infection point of 0.25OD600nm gave the greatest burst size
- Phage adsorption can differ based on culture system

Introduction

It is known that all microbes have a potential to mutate and reduce the effectiveness of antibiotics. Currently, there are around 50,000 deaths per annum in Europe and the USA, but by 2050, it is estimated that antimicrobial resistant (AMR) bacteria will cause around 10 million deaths per annum due to the misuse of antibiotics; however, their heavy use continues to this day (O'Neill, 2014; De Kraker et al, 2016; Josovsky et al, 2016). Nevertheless, within the past two decades, the use of bacteriophages have been exploited for the treatment of AMR bacteria due to the shocking rate of increase in resistant organisms, combined with the dearth of new methods to combat the infections (Gorski et al, 2016; Brussow et al, 2012; Kuchment 2012). Much of the current research focuses on the phage purification process as during the phage infection, bacterial endotoxins are released which can cause organ damage, inflammation and sepsis (Bourdin et al, 2014; Kleiner et al, 2015; Georgel, 2016).

However, there is a further gap in the knowledge that must be bridged; the optimised bioprocess and fermentation of bacteriophage. Such studies are scarce, and with phage therapy emerging into clinic and the numbers of clinical trials beginning to increase, it is vital that the fermentation parameters are

examined more closely to ensure that the future demand can be met. There have been 35 regulated clinical trials since 1995, 22 within the past 10 years, examining the effect of phage therapy against humans (clinicaltrials.gov).

Previous work has suggested that using an infection temperature of 28ºC is more beneficial to the phage infection compared to 37ºC and higher titres of phage can be attained (Greico et al, 2012; Ali et al, 2019). Additionally, other studies show that a lower infection temperature may be more beneficial for phage and viral propagation (Bleckwenn et al, 2005; Tokman et al, 2016). Within the few studies that consider fermentation, there have been no published studies into the conditions at which the point of infection takes place. This leads to the question of how to handle the culture between the pre-culture of host bacterium, at 37ºC, and the point of bacteriophage infection, with the infection taking place at a reduced temperature, in order to examine the effects on achievable phage titres, bacteriophage kinetics, adsorption rates and burst size.

Materials And Methods

Bacterial and phage strains

E. coli B and T4 bacteriophage were purchased from University of Reading. *S. aureus* and bacteriophage K (19685) (19685-B1) respectively were purchased from ATCC.

Media and growth conditions

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich, Irvine, UK. Luria broth (LB) media was used for growth of *E. coli* and T4 phage whilst brain heart infusion (BHI) media was used for growth of *S. aureus* and phage K. *E. coli* cultures were agitated at 225rpm whilst *S. aureus* cultures were agitated at 150rpm. For long term storage, bacteria were stored in a 20% glycerol solution at -80ºC whilst phage were stored at 4ºC but for long term storage, phage were kept in a 50% glycerol solution at -80ºC (Fortier & Moineau, 2009). T4 phage was infected at $0.25OD_{600nm}$ at a multiplicity of infection (MOI) 2.5 for 3 hours (225rpm) whilst phage K was infected at $0.25OD_{600nm}$ at an MOI 0.1, for 4 hours (150rpm).

Shake flask analysis

A single colony of each bacterium was inoculated in 20ml media overnight in a shake flask in a shaking incubator (Wolflabs midi shaking incubator SQ-4020). After 16 hours, the culture was diluted to an optical density (OD_{600nm}) of 0.05 with a Shimadzu biospec mini spectrophotometer. The culture was grown at 37ºC with shaking until it reached an optical density of $0.25OD_{600nm}$ and infected with phage.

Three methods of temperature reduction were examined to create differences in temperature reduction profile at the point of infection as follows. *Method 1*: the culture was placed into a shaking incubator at 37ºC and the temperature of the incubator reduced to 28ºC during the early stages of infection. *Method 2*: the culture was maintained at room temperature (22ºC) whilst the temperature of the shaking incubator

was reduced to 28ºC. *Method 3*: the culture was placed into a static incubator at 28ºC. Culture temperatures were monitored to record the temperature reduction profile over time. Enumeration, adsorption and burst analyses of early infection were carried out according to the methods described below.

Bioreactor analysis

A 5L Biostat B Plus stirred-tank bioreactor (Sartorius, Göttingen, Germany) was used at 3L working volume and with dO_2, maintained at 100%, pH maintained at 7.0 and an impellor used for agitation at 150rpm (*S. aureus*) or 225rpm (*E. coli*). A single colony of host bacterium was inoculated in 30ml medium (1% working bioreactor volume) and grown for 16 hours in a shake flask under standard conditions. This 1% volume culture was used as the inoculum for the bioreactor and grown to 0.25 OD_{600nm} at 37ºC. The bioreactor culture was then infected with phage: T4 phage was infected at an MOI 2.5 for 3 hours with 225rpm stirring, whilst phage K was infected at an MOI 0.1 for 4 hours with 225rpm stirring. These conditions were previously determined for achieving high titres at 3L scale (Ali et al, 2019). Immediately upon infection, the temperature of the bioreactor was set to reduce to 28ºC. Culture temperatures were monitored to record the temperature reduction profile over time. Adsorption, burst analyses and harvest phage titre enumeration were all carried out according to methods described above.

Phage purification

At the point of harvest, shake flask cultures or the equivalent volume from bioreactor cultures were centrifuged at 4,600g for 10 minutes and filtered using a 0.22µm filter (Millipore, Watford UK). Phage were concentrated using a 20% PEG-8000 overnight at 4°C. The following morning, the culture was centrifuged at 4,600g for 1 hour and the supernatant aspirated. The pellet was resuspended and stored in 1ml sterile media, LB for T4 phage or BHI for phage K. Harvest phage titre was enumerated by plaque assay described below.

Enumeration of phage

Phages were enumerated using the plaque overlay assay. Briefly, a 20ml overnight culture of host bacteria was centrifuged and re-suspended in 3ml fresh media and added to 5ml 0.6% LB bacteriological agar for T4 phage or 0.7% BHI bacteriological agar for Phage K. The mixture was then poured onto a fresh agar plate and the phage were spotted onto the top agar at appropriate dilutions (Bourdin et al, 2014; Pallavali et al, 2017). All shake flask and bioreactor experiments were carried out in triplicate and enumerated by triplicate plaque assays.

Adsorption analysis and burst size measurements

Adsorption: sacrificial shake flasks were prepared in duplicate for each of the methods under investigation such that adsorption measurements were taken at the point of infection and every 30 seconds from the point of infection to 5 minutes post infection (20 sacrificial flasks per method). For

each measurement, a 1ml culture sample was filtered using a 0.22µm filter and enumerated using the plaque assay technique (duplicate plaque assays per sample). For 3L Bioreactor cultures samples were taken directly from the bioreactor at the same time points during early infection and enumerated using the same method (triplicate experiments, single samples per time point, duplicate plaque assays per sample).

Burst size: experiments in shake flasks and bioreactors were performed in triplicate with 1ml samples taken at the point of infection and 30 minutes post-infection to determine the increase in phage after a single infection cycle i.e. burst size (Briggiler Marcó et al, 2015). Samples were filtered using a 0.22µm filter and enumerated using the plaque assay technique (triplicate plaque assays per sample).

Statistical tests

All statistical analysis was performed using IBM SPSS 23. Paired t-tests were used to calculate significance with a p value <0.05 considered to be statistically significant.

Results And Discussion

A reduction in temperature was previously shown to improve phage titres in bioprocessing (Greico et al, 2012, Ali *et al*, 2019), with temperature reduction hypothesised to aid efficiency in viral protein synthesis (Bleckwenn et al, 2005). However, the effect of the method of temperature reduction at the point of infection remains unclear. By examining three methods of temperature reduction (methods 1-3) this study aimed to provide insight into culture maintenance at the point of infection for improved infection kinetics and phage titres in bioprocessing. Within the study, host cell density (~$1x10^7$-$1x10^9$ cfu/ml) and a range of reduced temperatures (22.3-28ºC) at the point of infection were also investigated for effects on infection kinetics and harvest titres. Additionally, experiments in stirred tank bioreactors provided an insight towards scale-up of phage bioprocesses.

Small scale evaluation of temperature reduction method

In bench scale phage infection experiments, the simplest method of applying temperature reduction is to allow the shaking incubator to reduce to the desired temperature once culture infection has taken place. However, this will not be an applicable method for a scalable bioprocess for large scale production of phage.

To provide information on the best method for temperature reduction at bench scale (20ml shake flask cultures) with a view towards informing development of scalable culture processes, three methods of culture maintenance during early infection and temperature reduction were investigated. The methods of temperature reduction were Method 1, culture maintained in the shaking incubator during temperature reduction (from 37 – 28ºC); Method 2, culture maintained at room temperature (22ºC) during shaking incubator temperature reduction (to 28ºC); and Method 3, culture maintained in a static 28ºC incubator during shaking incubator temperature reduction (to 28ºC). These methods were applied to two different

host-phage culture processes; *E. coli* – T4 phage and *S. aureus* – Phage K. The temperatures of both the cultures and the environment were monitored during early infection and the results are shown in Figure 1.

Under Method 1, the shaking incubator was shown to take 8 minutes to reduce from 37ºC to 28ºC, whereas the culture temperatures of both organisms reached the desired 28ºC temperature 14 minutes after infection and stabilised by 18 minutes post-infection (Fig 1, A-B). The observed slower rate of culture temperature reduction compared to environment reduction was expected due to the heat transfer from incubator to shake flask culture. Under Method 2, as expected culture temperature reduced more rapidly when maintained at room temperature during early infection. These cultures achieved desired culture temperature at 5 minutes post-infection (Fig. 1 C-D) and were then transferred back to the shaking incubator to complete the infection cycle. Under Method 3, when cultures were maintained in a pre-set 28ºC static incubator for a slower and more controlled temperature reduction profile compared to method 2, the desirable culture temperature was achieved at 11 minutes post-infection. Temperature shock to the culture can have a negative impact on the host cells or phage and therefore it may be more desirable to have a more controlled initial reduction in temperature (Jończyk et al, 2011).

Previous work has shown that by adapting the conditions at which the phage infection takes place; MOI, agitation, time and the temperature of infection, a consistent phage titre of ~$5x10^{13}$ and $1x10^{12}$ pfu/ml can be achieved for T4 phage and phage K respectively (Ali et al, 2019). The data presented in figure 2 shows increases in harvest titre can be achieved by altering the reduction temperature profile during early infection i.e. method 3 significantly improved harvest phage titres for both host-phage systems. Method 3 consistently achieved titres of $7.89 \times 10^{13} \pm 1.9 \times 10^{13}$ pfu/ml and $1.03 \times 10^{13} \pm 1.77 \times 10^{13}$ pfu/ml for T4 and phage K respectively. There was a statistically significant increase in harvest titres between each method (1-3) for T4 phage and method 2-3 only for phage K ($p<0.05$). It is unclear as to why reducing the temperature at the point of infection improves phage titre across two different host-phage systems. However, to elucidate some of the underpinning mechanisms at the point of infection that may be affecting these differences in final titre and process yield, the phage infection kinetics in terms of adsorption and burst size were assessed.

Within bacteriophage fermentation literature, titres of 1×10^{12} pfu/ml are currently some of the greatest achievable levels (Bonilla et al, 2016). Previous studies have shown that optimising conditions at which phage fermentation takes place can improve the titre achievable (Greico *et al*, 2009, Greico *et al*, 2012). However, by examining the conditions at the point of infection this study has shown a further increase by 1 order of magnitude in harvest phage titres depending on the method of initial temperature reduction during early phage infection (method 3). These methods and titre gains are applicable to small scale fermentations and will help to inform scalable fermentation. Whilst no previous work has examined a similar mechanism, a hypothesis for the improvement in titre may be if the optimal temperature for T4 and phage K infection is 28^{o}C, maintaining the culture at the most desirable temperature could improve titre by slowing intracellular processes involved in viral propagation, however this is yet to be determined experimentally. Additionally, by preventing a shock to the culture i.e. significant temperature drops which could affect host cell metabolism, efficiency of replication and the ability of phage to propagate. This

hypothesis is somewhat supported by the data shown here where reducing the potential for cellular shock using a slower and more controlled temperature reduction profile improved phage titre, but requires further investigation to determine the biological mechanism of action.

Adsorption and burst analysis

An important study in the kinetics of phage infection is the adsorption of the phage to the host cell and the burst size. It is known that phage adsorption is dependent on the culture conditions (Zaburlin et al, 2017) and differences in phage adsorption kinetics may offer some mechanistic explanation for the differences in titre achieved with the various methods of temperature reduction at the point of infection. Figure 3 shows the adsorption of phage to their host cell for each of the infection methods (1-3). Figure 3A shows that each of the methods had a negligible difference in the adsorption of T4 phage for the first 6 minutes. Thereafter, a statistically significant difference was observed in the rate of adsorption between methods 2 and 3 compared to method 1 where the culture was agitated throughout early infection. There was very little difference between the cultures without agitation (methods 2 and 3) that were held at room temperature or in the static 28ºC incubator respectively. Figure 3B shows a greater degree of variation was observed in the phage K adsorption compared to the T4 phage adsorption. A significantly greater rate of adsorption was observed in incubated cultures (methods 1 and 3) compared to cultures held at room temperature (method 2) from 3 minutes post-infection onwards ($p<0.05$). Agitation appears play less of a role in influencing phage K adsorption compared to T4 phage adsorption, with no significant difference observed between 7-10 minutes post-infection between methods 1 (agitated) and 3 (static) for phage K whereas only the agitated method influenced T4 phage adsorption.

A further important study in bacteriophage kinetics is the burst size, the increase in phage after the bacterium has been infected with phage (Wang, 2006). Table 1 below shows the average burst size achieved from each phage infection for each of the three temperature reduction methods. Burst size for method 3 was statistically significantly increased compared to methods 1 and 2 ($p<0.05$) for both phage-host systems. A lower average burst was observed for method 2 compared to method 1 for both phage-host systems although only statistically significant for phage K. This lower burst may be related to reduce control in the temperature reduction profile and possible temperature shock to the culture with method 2 where cultures were removed from 37^{o}C incubation to room temperature (22^{o}C). The temperature reduction during early infection is more gradual for method 1, achieving the desired 28^{o}C culture temperature over 14 minutes compared to 5 minutes for method 2.

Fister et al (2016), previously examined the effect of environmental factors on the adsorption and yield of phage, their data showed how reducing the pH or the addition of salts can negatively influence the adsorption rate of *Listeria* phage P-100 although it wasn't significant. The data presented in this study shows that culture temperature during early infection and the method of exposure to a change in temperature can influence adsorption rates in multiple host-phage systems. Although there are differences between the level of influence of temperature on the different host-phage systems, for example different burst sizes for T4 vs. phage K with method 3 temperature reduction and differences in

response to agitation for T4 phage. The data shows evidence that a gradual reduction in temperature positively influences adsorption rate and burst size during early infection. Other environmental factors, such as MOI, pH and agitation may also have a significant effect on the phage-host adsorption (Silva & Sauvageau, 2014).

Infection point

An additional aspect when assessing the point of infection is the growth of the host and the point in the host's growth cycle at which phage infection takes place. It is established in literature that phage infection should be introduced within early logarithmic growth phase and OD_{600nm} 0.2-0.4 is commonly used (Bryan et al, 2016, González-Menéndez, et al 2018).

Higher host cell densities result in lower harvest phage titres being achieved, thought to be related to fewer phage interactions with host cells and reduced levels of infection, whilst lower host cell densities are too dilute and do not contain sufficient cell numbers to effectively propagate phage (Kasman et al, 2002, Kick et al, 2016). However, it is unclear whether there is an optimal point within this established cell density range nor whether this potential optimal point differs between host – phage systems. Establishing standardised methodology with control points such as at the point of infection will help to inform standardised bacteriophage manufacturing processes with minimal variation in desirable outputs. *E. coli* and *S. aureus* cultures were grown to a range of optical densities within and outside the pre-determined range (covering the growth cycle) before phage infection with T4 and phage K respectively, to assess the effect of infection point and the effect on final phage titre and burst size in moving towards a more standardised infection point for these systems.

Figure 4 shows the greatest titre for each phage was observed at an infection point of $0.25OD_{600nm}$ and the titre achieved was statistically significant higher than the titres achieved from adjacent infection points, $0.3OD_{600nm}$ and $0.2OD_{600nm}$, for both T4 ($p<0.001$; $p<0.003$) and phage K ($p<0.004$; $p<0.004$) and for all other infection points. At $0.25OD_{600nm}$, a titre of $2.2x10^{13}$ and $2.5x10^{11}$ was achieved for T4 and phage K respectively. The phage burst sizes were calculated for each infection and show an expected clear trend in alignment to the titre results with an increase in burst related to increased output titre. Distinct peaks in burst size aligned to the greatest phage titres achieved at $0.25OD_{600nm}$ for each phage. Statistically significantly lower burst sizes were achieved between 0.25 and $0.2/0.3OD_{600nm}$ and 0.25 - $0.05/0.5/1OD_{600nm}$ for both phage, $p<0.05$. The results show the importance of host cell density at the point of infection upon maximising output titres and that parameters such as host cell density may be comparable in their optimum for multiple host-phage systems. Although individual phage may respond differently to the point of infection, it is plausible to consider that $0.25OD_{600nm}$ could be used as a standardised host density at the point of infection for *E. coli* or *S. aureus* host production systems. Whether a standardised host density could be applied more widely to further species or host-phage systems would require experimental determination.

An additional experiment was carried out to determine the effect of phage infection once the host culture had stabilised at 28^{o}C (under standard conditions phage inoculation occurs immediately prior to temperature reduction). Previously, figure 1 showed that under Method 1, where cultures were kept in a shaking incubator during temperature reduction, it took 14 minutes for cultures to stabilise at 28°C. During this experiment, 0.25OD_{600nm} cultures were inoculated with phage once they were stable at 28°C following Method 1 temperature reduction. The experiment was carried out in triplicate and with duplicate phage plaque assay titre measurements (n=6) and single burst size measurements (n=3). The results showed there was no significant improvement on the T4 or phage K titre, $8.17x10^{12}$±$4.05x10^{12}$ and $2.67x10^{12}$±$8.73x10^{11}$ pfu/ml. T4 phage gave a more variable and reduced burst size of 107.7±19.9 whilst phage K gave a reduced burst size of 72.1±6.2 when compared to the bursts achieved at 0.25OD_{600nm} above (Fig. 4). The results suggest there is no advantage in postponing phage inoculation for culture temperature reduction to stabilise and taken together with the previous phage adsorption results (Fig. 3) that inoculation followed by temperature reduction may be more beneficial to the very early phage infection process.

Scale up model evaluation of the temperature kinetics

The small-scale shake flask experiments allow for high throughput approach and fewer resources when compared to larger stirred-tank bioreactor systems. However, small-scale shake flasks are not fully representative of the industrial-scale systems that will be employed for the production of bacteriophage, given the differences in fermentation dynamics relating to increased volume and impellor-based agitation. Fermentation of bacteriophage is moving towards larger scale STR cultures rather than shake flask culture (Agboluaje & Sauvageau, 2018) as well as other scalable methods and as such it is important to examine small scale outcomes informing bioprocess development in relevant scalable culture systems. This type of study is also important to examine the differences between the culture systems in terms of bioprocess outcomes to generate knowledge on the advantages and limitations of the translation.

Although the greatest titre was achieved using Method 3 in the small-scale experiments, this method of static incubation is not practicably replicated in a 5L bioreactor. However, further experimentation beyond the scope of this study is required to investigate the effects of stirring and agitation dynamics on early phage infection. The Method 3 results suggest that suspending agitation during early infection / temperature reduction may be beneficial for phage adsorption, burst and related harvest titres in scalable bioreactor cultures.

Initially, the process from the small-scale Method 1 experiments was replicated in a scaled up stirred tank bioreactor model. Bioreactor cultures were maintained at 3L working volume with 100% dO_2, pH7.0 and 225rpm / 150 rpm impellor stirring for *E. coli* / *S. aureus* respectively. Cultures were infected with phage at 0.25OD_{600nm}, MOI of 2.5 / 0.1 respectively and immediately cooled from 37ºC to 28^{o}C. Figure 5 shows the difference in phage adsorption during early infection between the shake flask and bioreactor culture systems. The shake flask cultures generally gave a greater rate of adsorption for phage K (Fig. 5B),

whereas T4 phage showed initially there was a higher level of free phage in the STR cultures but after 7 minutes there was less free phage in the shake flask model (Fig. 5A). The experiments were completed in duplicate and each sample enumerated with duplicate plaque assays. Figure 5 C and D show the temperature reduction in the stirred tank reactor culture. They show that the culture took around 14 minutes to reduce to the desired 28^{o}C temperature. The action of the water jacket and agitation will allow the heat transfer throughout the culture and consequently the reduction in temperature. Given that ultimately, manufacturers may look towards continuous culture, future experiments may benefit from replicating this process in a scale up continuous system, i,e feeding cells into a 28^{o}C reactor.

It was important to note the final titres achieved in the experiments in the 5L bioreactor. The experiment was completed in duplicate with triplicate plaque assay measurements and duplicate burst size measurements. There was a statistically significant increase in titre between method 1 in shake flasks and the 5L scale up model for T4 phage, 5.11 x 10^{12} ± 1.5 x 10^{12} pfu/ml and 4.33 x 10^{14} ± 9.4 x 10^{13} pfu/ml $p<0.01$ using an ANOVA whilst phage K gave a statistically significant increase between method 1 in shake flasks and the 5L scale up model for phage K 1.1x10^{12}±1.02x10^{12} and 8.5x10^{12}±8.99x10^{12} pfu/ml respectfully, $p=0.001$ using an ANOVA. This increase in titre between the scale up model and the shake flasks can be accounted for by the change in system used. The stirred tank system offers the advantage of a more controlled environment that the infection can take place in which will consequently an increase in titre is seen. Previous work has shown the improvement in titre between the two culture systems using the same conditions, given the more controlled environment (Ali et al, 2018). Figure 1 showed that the culture took around 14 minutes to reduce from 37^{o}C to 28^{o}C and stabilise, similar to the scale up model. The graphs show a statistically significantly higher rate of adsorption in the first 6 minutes for T4 phage in the shake flask compared to the 5L bioreactor, figure 5A and B. It is interesting to note this as although the conditions were kept the same, the agitation between the culture systems will differ despite the same agitation rate being used, 225rpm *E. coli*, 150rpm, *S. aureus*. Additionally, phage K showed a statistically significantly higher rate of adsorption in the shake flask after 2 minutes of infection between each time point $p<0.05$ using a paired t test. The sheer damage to the host cells and difference in mixing patterns seen in the STR due to the impellor may cause differences in the adsorption between systems (Borys et al, 2018). The graphs show that after 10 minutes, around 99% of phage had adsorbed to their host cell. The difference in the mixing between shake flasks and STR, may affect the adsorption of phage to their host organism and it was interesting to note the differences seen between the culture systems. The harsh agitation caused by impellors may account for the reduced higher free phage in the stirred tank culture despite the same agitation rate used across the systems (150rpm – *S. aureus*/phage K, 225rpm *E. coli*/T4 phage).

The duplicate experiment in the STR gave an average titre of 4.33x10^{14} ± 9.4x10^{13} pfu/ml and 4x10^{12} ± 1.41x10^{12} pfu/ml for T4 phage and phage K respectively. The advantage of using a scale up model is two-fold. Firstly, the increase in yield between the shake flask and STR model within their respected bioprocesses and secondly the improvement in maintenance of conditions which improves the overall bioprocess. This can be seen from the titre achieved as in the shake flask model, figure 2, the greatest

titres achievable were $7.89x10^{12} \pm 1.96x10^{12}$ and $1.03x10^{13} \pm 1.77 x10^{12}$ pfu/ml for T4 phage and phage K respectively. It is interesting to note the burst size achieved in table 1. In the 5L bioreactor, T4 gave a duplicate average burst size of 97.45 ± 7.3 whilst phage K gave an average burst size of 106.1 ± 8.4. The statistically significantly increased burst in the shake flask whilst kept in a static incubator, compared to the 5L for T4 is interesting to note and could be accounted for in the difference in mixing or pH control. Although no significant difference was seen in the phage K burst, a higher titre was achieved in the 5L culture compared to the shake flask culture. The maintenance of the conditions in the STR would allow an overall higher titre to be achieved when the experiment was run to completion. One study has previously shown that reducing the temperature of infection gave a higher titre at 25^{o}C compared to 37^{o}C (Shan *et al*, 2014). Additionally, Vasina & Baneyx (1997) showed that reducing the temperature from 37^{o}C to 30^{o}C after *E. coli* was grown to mid log phase allowed greater protein production to be found. Hypotheses were given stating that reducing the temperature allowed metabolic processes to be extended and therefore the host organisms could replicate for longer periods of time. Monoclonal antibody titres have also been shown to improve when grown at 31^{o}C compared to 37^{o}C (Boigard *et al*, 2018). Previous work has shown significantly improved yields using an STR over shake flask due to pH and oxygen control. Changes in pH will affect the ability of the host organisms to propagate themselves and the phage (Nobrega *et al*, 2016). Moreover, there will be differences in the mass transfer within the culture, between the shake flask and 5L STR, which may also cause gradients again limiting the propagation ability which can be mitigated in bioreactors as they have less mass transfer limitations (Klockner & Buchs, 2012). Given the length of the culture and the improved control in the process throughout, compared to the shake flask model, it may help to explain why greater titres and burst sizes were seen in the STR.

Conclusion

Perhaps the most understudied area within bacteriophage research is its production. Previous research has shown that phage titres can be improved by improving the overall culture conditions. This study examined the conditions at which the T4 and phage K cultures are kept at, at the point of infection. The data presented here shows that maintaining the culture in a static incubator at 28^{o}C, whilst the shaking incubator cools down to 28^{o}C, from the pre culture temperature of 37^{o}C, a significantly improved phage titre can be achieved. Additionally, the burst size is also increased in shake flasks. Whilst minimal differences were seen in the adsorption at each condition, it was interesting to note the differences seen between T4 and phage K. Furthermore, the experiment also studied the point of phage infection. Whilst many infect cultures between 0.2-0.4OD600nm, it was important to show at what infection point the greatest titres and burst sizes were achieved and how the final titres could be statistically significantly improved. This study has shown a further degree of optimisation of two phage bioprocesses and highlights the need to study every part of a bioprocess to improve the final product output. Moreover, an investigation was made into STR phage work, which is severely lacking in the current field. However, future phage therapy will depend on the ability of phage propagation in larger reactors to produce higher yields. Although some of the conditions investigated could not be directly translated into the STR due to

differences in the mechanism for cooling, the maintenance of the best conditions in the STR would allow for higher titres to be achieved in experiments run to completion. Further investigation is therefore required into establishing optimal cooling profiles during infection of larger scale cultures and the optimal parameters at infection point for continuous cultures. Future work, must also consider similar studies for other phage as it is well known phage infection kinetics differ between phage-host systems.

Declarations

Ethics approval and consent to participate – Not applicable

Availability of data and materials – All data generated or analysed during the study are included in this article

The authors declare they have no competing interests

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J. Ali carried out the experimental work. E. Ratcliffe & Q. Rafiq assisted with data interpretation and contributed to the writing of the manuscript

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Tables

Table 1. Phage burst size with different culture temperature reduction methods applied during early phage infection. Error is shown by 1 standard deviation of single burst size measurements from duplicate experiments.

Method	Condition	Burst size (± 1SD)	
		T4	K
1	Culture kept in shaking incubator during temperature reduction (from 37 – 28°C)	80.7 ± 9.3	79.5 ± 6.5
2	Culture kept at room temperature (~22°C)	65.6 ± 9.5	66.6 ± 4.05
3	Culture kept in static incubator (~28°C)	143.75 ± 2.2	96.9 ± 3.1

Figures

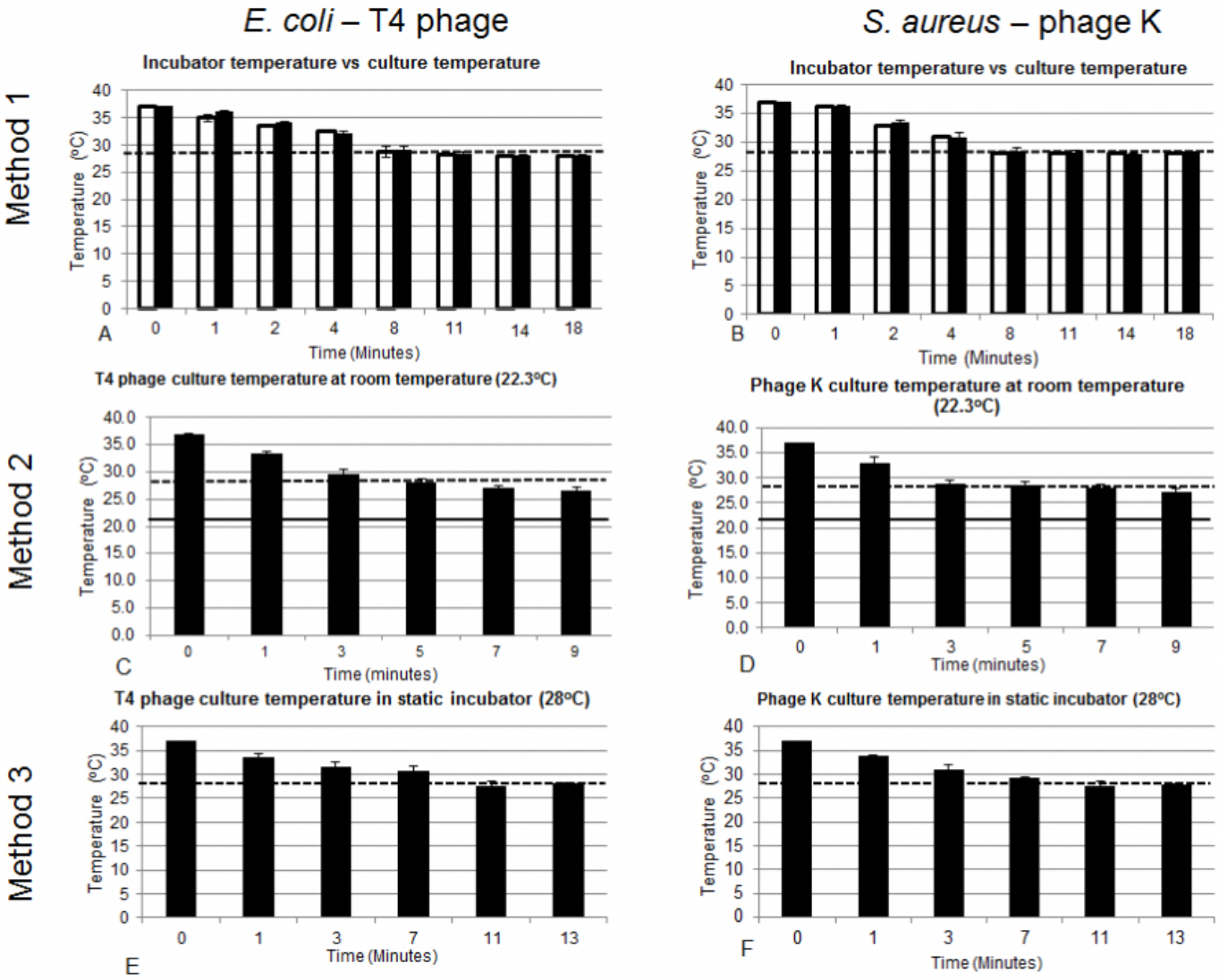


Figure 1

A comparison of temperature reduction methods on achieving desirable culture temperature during early phage infection. Method 1 (A-B): culture maintained in the shaking incubator during temperature reduction (from 37 – 28ºC) for E.coli – T4 Phage (A) and S. aureus – Phage K (B). The culture reached the desired temperature at 8 minutes. Method 2 (C-D): culture maintained at room temperature (22ºC) during shaking incubator temperature reduction (to 28ºC) for E.coli – T4 Phage (C) and S. aureus – Phage K (D). The cultures reached the desired temperature at 5 minutes. Method 3 (E-F), culture maintained in a static 28ºC incubator during shaking incubator temperature reduction (to 28ºC) for E.coli – T4 Phage (E) and S. aureus – Phage K (F). The culture reached the desired temperature at 11 minutes. In all cases phage infection started at time zero, culture temperatures were monitored and shown as black columns on graphs. Graphs A and B also show the temperature monitoring of the shaking incubator environment (white columns). Graphs C and D also show the average room temperature (22.3ºC ± 0.1ºC, solid line) and graphs C-F all show the desirable culture temperature (28ºC, dashed line).

All experiments were performed in triplicate over three separate days to account for any environmental or equipment variation (which was minimal). Error bars indicate 1 standard deviation of each triplicate experiment.

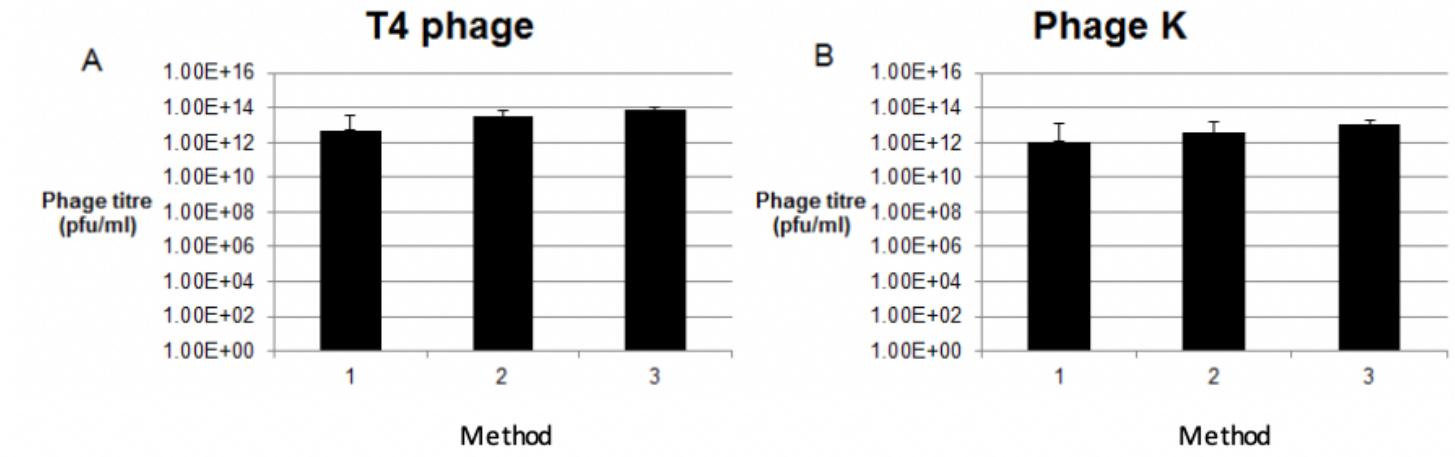


Figure 2

A comparison of temperature reduction methods on T4 phage (A) and Phage K harvest titres (B). Method 1: culture maintained in the shaking incubator during temperature reduction (from 37 – 28°C); Method 2: culture maintained at room temperature (22°C) during shaking incubator temperature reduction (to 28°C); Method 3: culture maintained in a static 28°C incubator during shaking incubator temperature reduction (to 28°C). Experiments were performed in triplicate with each run enumerated with triplicate plaque assays (n=9). The error bars represent 1 standard deviation of triplicate experiments.

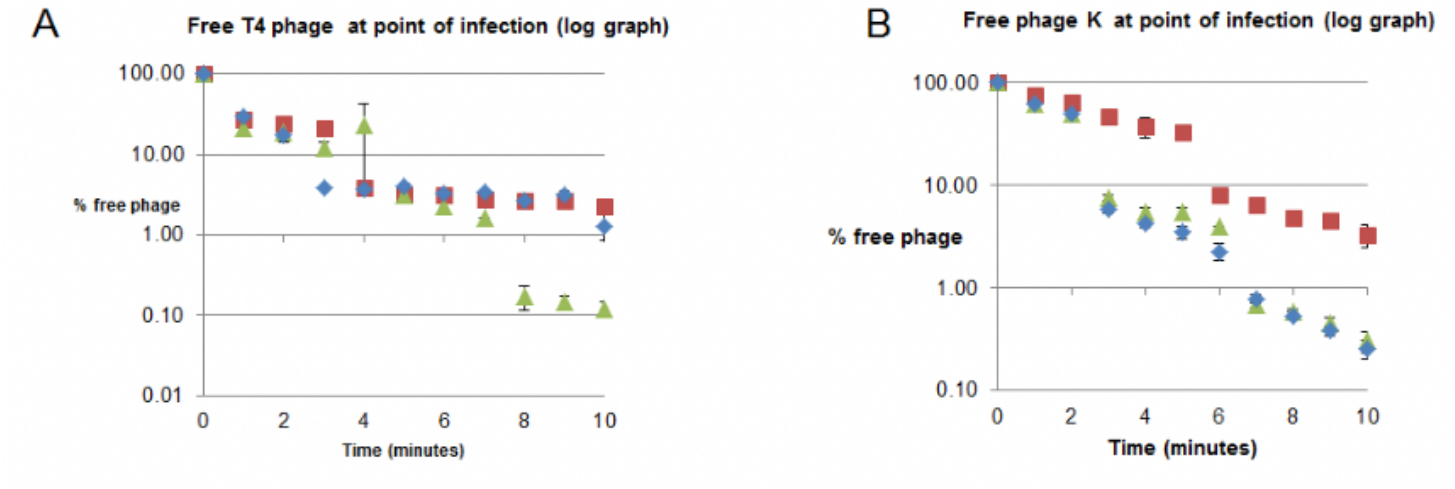


Figure 3

Phage adsorption to host cells with different culture temperature reduction methods applied during early phage infection. Free phage (%) after infection (at time zero) when culture temperature was reducing to

28°C in a shaking incubator (method 1 - green triangles); held at 28°C in a static incubator (method 3- blue diamonds); or held at 22°C room temperature (method 2 - red squares) for T4 phage (A) and phage K (B). Error bars represent 1 standard deviation of duplicate experiments with each experiment enumerated with duplicate plaque assays, n=4 total.

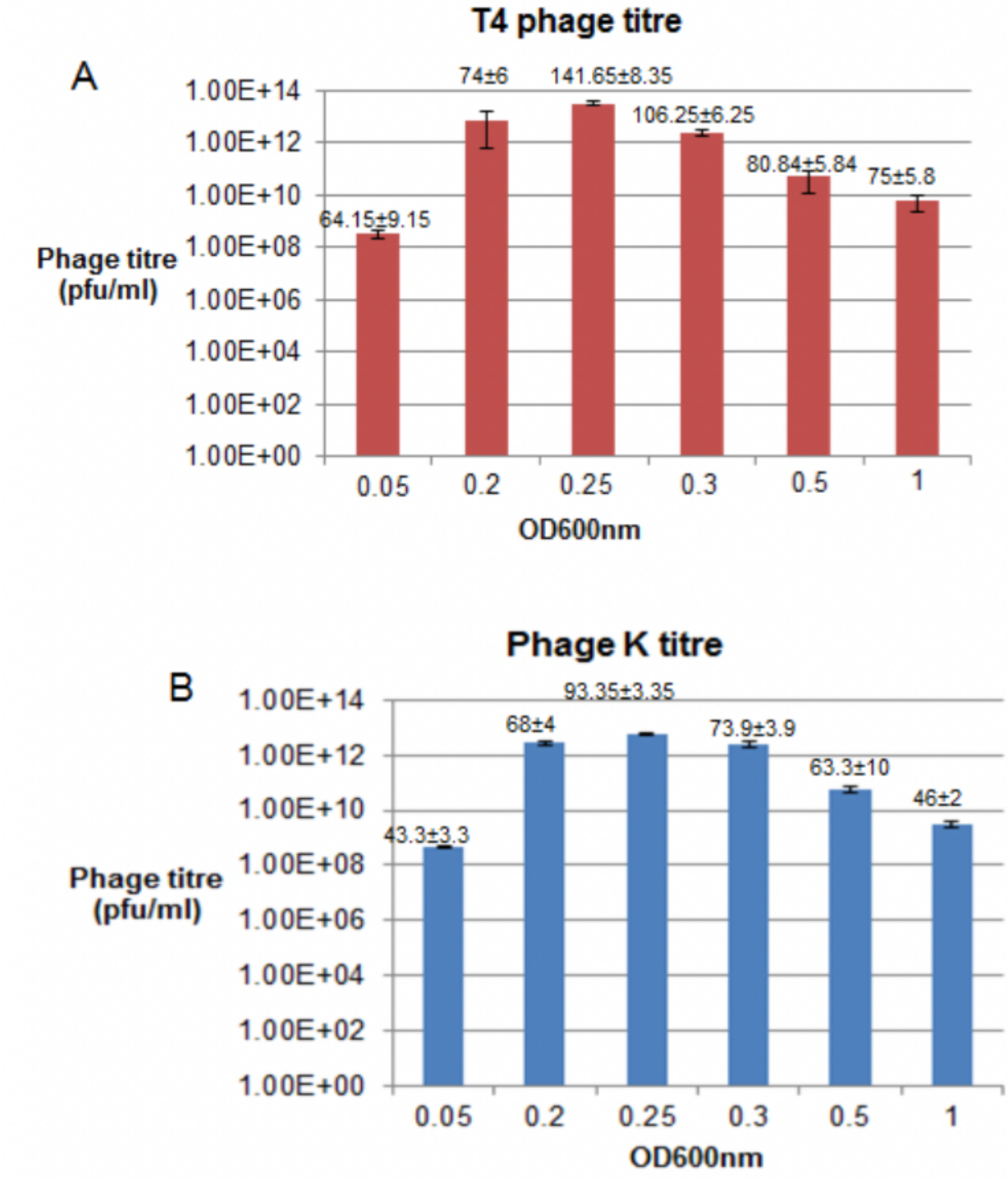


Figure 4

Effect of varying the bacteriophage point of infection in the host growth cycle on achievable phage titre. Phage titre (plaque forming units / ml) after infection under standard shake flask conditions for T4 phage (A, 0.25OD600nm, MOI 2.5, 28°C, 225rpm, 3 hours (Method 1)) and phage K (B, 0.25OD600nm, MOI 0.1, 28°C – Method 1, 150rpm, 4 hours (Method 1)). Error bars represent 1 standard deviation of duplicate experiments with each experiment enumerated with duplicate plaque assays, n=4 total. Values above each bar represent the mean phage burst size ± 1 standard deviation from duplicate experiments.

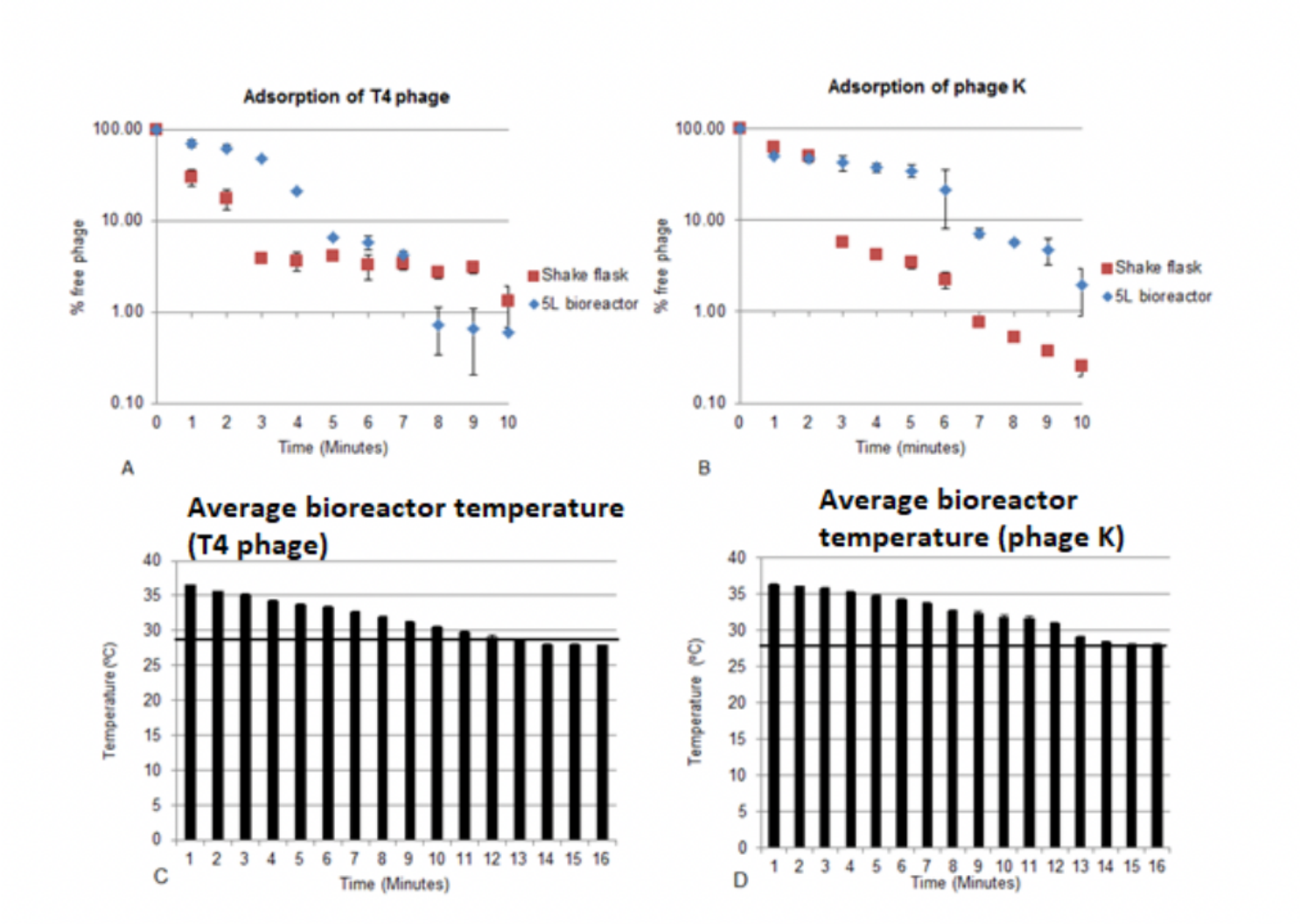


Figure 5

Phage Adsorption to host cells during early phage infection temperature reduction in scalable bioreactor cultures. Free phage (%) after infection (at time zero) when culture temperature was reducing to 28°C in shake flasks (squares) and the 5L bioreactor (diamonds) for T4 phage (A) and phage K (B). Error bars represent 1 standard deviation of duplicate experiments with each experiment enumerated with duplicate plaque assays, n=4 total. Graphs C and D also show the temperature monitoring of the bioreactor cultures (3L working volume, average temperature from duplicate experiments) during reduction to the desirable culture temperature (28°C, solid line).