A scaled down model for the translation of bacteriophage culture to manufacturing scale

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Abstract

Therapeutic bacteriophages are emerging as a potential alternative to antibiotics and synergistic treatment for antimicrobial resistant infections. This is reflected by their use in an increasing number of recent clinical trials. Many more therapeutic bacteriophage are being investigated in pre-clinical research and due to the bespoke nature of these products with respect to their limited infection spectrum, translation to the clinic requires combined understanding of the biology underpinning the bioprocess and how this can be optimised and streamlined for efficient methods of scalable manufacture. Bacteriophage research is currently limited to laboratory scale studies ranging from 1-20mL, emerging therapies include bacteriophage cocktails to increase the spectrum of infectivity and require multiple large scale bioreactors (up to 50L) containing different bacteriophage – bacterial host reactions. Scaling bioprocesses from the millilitre scale to multi litre large scale bioreactors is challenging in itself, but performing this for individual phage-host bioprocesses to facilitate reliable and robust manufacture of phage cocktails increases the complexity. This study used a full factorial Design of Experiments (DoE) approach to explore key process input variables (temperature, time of infection, multiplicity of infection, agitation) for their influence on key process outputs (bacteriophage yield, infection kinetics) for two bacteriophage – bacterial host bioprocesses (T4 – *E. coli*; Phage K – *S. aureus*). The research aimed to determine common input variables that positively influence output yield and found that the temperature at the point of infection had the greatest influence on

bacteriophage yield for both bioprocesses. The study also aimed to develop a scaled down shake flask model to enable rapid optimisation of bacteriophage batch bioprocessing and translate the bioprocess into a scale up model with a 3L working volume in stirred tank bioreactors. The optimisation performed in the shake flask model achieved 550-fold increase in bacteriophage yield and these improvements successfully translated to the large scale cultures.

Key words: bacteriophage, propagation, antimicrobial resistance, bioprocess, scalable manufacture

Introduction

Antimicrobial resistance is increasing at an alarming rate with few treatment options for related diseases and a dearth of novel solutions (Davies *et al*, 2013). The nature of resistance is highly complex with a multitude of factors that each contributes to resistant organisms forming. Antimicrobial resistant bacteria can develop because of self-inflicting factors such as patients not completing a course of antibiotics or saving and sharing antibiotics amongst others (Goldsworthy *et al*, 2009). Exposure to antibiotics, when their use will bring no additional benefit may also allow the formation of resistant cells (McNulty *et al*, 2007). Additionally, transfer of genetic material between bacteria which codes for resistance genes plays a major role in the development of resistant bacteria (Burmeister, 2015). It is estimated that mortality rates will rise to over 10 million per annum by 2050 due to infections caused by resistant bacteria and therefore, novel strategies are needed to tackle antimicrobial resistance (AMR). Additionally, it is predicted that AMR will impact global GDP by over $100 trillion by 2050 (O'Neill, 2014). Studies have shown that approximately 54% of predominant *E. coli* strains are resistant to at least one antimicrobial drug (Tadesse *et al*, 2012). More recently, a study was carried out where 137 *Escherichia coli* (*E. coli*) clinical isolates were tested for resistance to 11 commonly used antibiotics and showed 50 of the isolates tested were resistant to 10 of the 11 antibiotics, highlighting the urgent requirement for alternative therapies (Olorunmola *et* al., 2013). More recently, a study sampled 862 clinical isolates of *E. coli* from a variety of animals

including chickens, pigs and cows and found that 94% of strains were resistant to 1 drug and 83% were resistant to 3 antimicrobial classes (Yassin *et al*, 2017).

At the beginning of the 20th century, bacteriophages (phages) were believed to have the potential to act as antimicrobial agents, although it is only within the past two decades that their true potential has emerged (Mandal *et al*, 2014). Bacteriophages are the most abundant organisms in the biosphere with an estimated 1 x 10^{29} phages on Earth. They can be easily isolated from rivers and sewers and cultured through infecting their host strain, and then purified *via* centrifugation and simple filtration (Millard *et al*, 2011). Phages initially showed potential in treating bacterial infections in the early 20th century, however, with the discovery of penicillin in 1928 and the advent of the antibiotic age, phage therapy research did not progress due to the lack of medical need. Although early trials using phages against *pneumococci* and *Corynebacterium diphtheria* showed promise in the 1920-1930s, poorly controlled trials and inconsistencies within results led to discontinuation of phage therapy clinical trials (Whittebole *et al*, 2014, Pires *et al*, 2016). By the 1940s, Western medical regulations dampened enthusiasm on phage therapy despite research remaining high in the former USSR. Between 1940-1950 there were around 2,000 studies published on phage therapy, compared to the near 20,000 studies using penicillin. In Eastern European countries, bacteriophage therapy is a tool used within modern medicine despite several major concerns regarding their safety (Nale *et al*, 2016). Some of the safety concerns have arisen because the production of phage requires infection and lysis of host bacteria which leads to the release of bacterial endotoxins. These endotoxins must be removed from the final culture before clinical use. During the lysis of the host cell, the bacterium releases newly formed phages and bacterial endotoxins, which must be removed from final product preparation due to their inflammatory properties that can cause organ damage, failure and sepsis. Although phage preparations can be purified from endotoxins to the levels required for regulatory approved clinical trials, the purification process is long and expensive and represents one of the main hurdles to success (Slofstra *et al*, 2006, Catalão *et al*, 2013, Georgel, 2016). Aside from safety, other disadvantages of phage therapy include their narrow host range which can limit their treatment potential and their poor distribution as they have no mechanism for movement and rely on random

interactions (Loc-carillo & Abedon, 2011). However over the past two decades Western medicine has regained interest in bacteriophage therapy with 35 carefully regulated clinical trials since 1995, 22 of which occurred in the past 10 years (clinicaltrials.gov). To date, there have been 5 clinical trials using phage against *E. coli* and 6 against *Staphylococcus aureus* (*S. aureus)*. Additionally, a PubMed recent literature search showed >1,000 journal articles were published in 2017 when searching for the combined terms "bacteriophage" and "therapy" (PubMed 2018, 2018).

With renewed interest and increasing levels of current research, phage therapies are emerging as potential tools against antimicrobial resistant infections (Bragg *et al*, 2014, Speck & Smithyman 2016, Lin *et al*, 2017). However in addition to the required improvements in phage purification, the standardisation and phage production process has not been widely explored. Throughout the literature there are references to the importance as well as the need for scale up, yet studies exploring large scale manufacture of phages are scarce (Warner, *et al*, 2014, lomtscher *et al*, 2017, Krysiak-Baltan *et al*, 2018). For successful translation of phage therapies into the clinic, scalable, robust and cost effective manufacturing processes are required to match the expected increased demand. This necessitates the identification of key process input variables (KPIV) and key process output variables (KPOV) such that optimisation strategies can be employed for improved bioprocess outputs, as well as for standardisation of common units of manufacture. Additionally, understanding the range of the KPIV used allows greater control over the final product (Ratcliffe *et al*, 2011).

The aim of this study was to determine common KPIV that could positively influence bacteriophage output yield and elucidate combined conditions at which the greatest phage yield could be achieved for two different bacteriophage bioprocesses (T4 – *E. coli*; Phage K – *S. aureus*). These organisms were chosen as suitable candidates given the previous use of T4 phage and the worrying rise in *S. aureus* infections (Sarker *et al,*2012). Using a full factorial Design of Experiments (DoE) approach a further research aim was to characterise the design space for each bioprocess in a scaled down shake flask model for high throughput analysis with validation of the result to ensure reliability before translating to large scale culture. The study focussed on developing the approach for scalable

batch bioprocessing which is currently employed in manufacture of phage for clinical trials with a view towards developing the research towards continuous bioprocessing in future studies.

Using a full factorial design allows a methodological approach to enable parallel analysis of multiple experimental factors whilst gaining insight into interactions between each factor and an estimation of the effects of each of the variables such as the contour plots seen in Figure 1 described and discussed below. Recently, Stuible *et al* 2018 showed the effectiveness of using DoE for high bacterial cell density for protein production. By examining their KPIV they determined the levels at which greatest protein and antibody production could be achieved. A further advantage of the full factorial approach in characterising a bioprocess design space is the ability to predict other areas where similar levels can be produced, which can be a powerful tool when increasing achievable scale.

KPIV investigated in the scaled down model were temperature, multiplicity of infection (MOI), agitation, and time of infection due to the potential impact they may have on the culture. They were used to determine levels of the input variables in combination that could significantly impact upon key process output variables based on output phage titre and measures of bioprocess yield (outputs vs. inputs). The temperature of infection has not been widely studied to date with minimal studies examining its effect on phage titre. Greico *et al*, (2012) showed that a reduction in temperature can improve the phage titre achieved, whilst Bleckwenn *et al* 2005 hypothesised that a reduction in temperature aids viral protein synthesis. Due to the phage infection mechanism, reducing the temperature may aid in the integration of phage DNA leading to an improvement in the production of phage and allow it to become more efficient thereby producing higher titres. MOI was investigated as a high MOI may cause negative feedback whilst using an MOI that was too low may increase the time for phage propagation (Bourdin et al, 2014, Heggen et al, 2014, Bryan et al, 2016, Alves et al, 2014, O'Flaherty et al 2005). Agitation was also investigated as mixing of the culture is vital for homogeneity of oxygen and pH in the culture to allow optimal growth of host cells (Bourdin et al 2014, Greico et al, 2012, Basdew et al 2012, Paul et al 2011). Finally, the time of infection was investigated, as although not previously studied, being able to decrease the time would have a significant impact on bioprocessing efficiency. Moreover, by allowing phage infection to take place

over a longer period, there is a risk that phage may bind to receptors on lysed host cells and therefore be lost during the filtration steps (Alves et al, 3024, Estrella et al, 2016, Choi et al, 2010).

Materials and methods

Bacterial and phage strains

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

Media and growth conditions

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich, Irvine, UK. Luria broth (LB) (miller) medium was used for growth of *E. coli* and T4 phage. *E. coli* was grown at 37°C with constant agitation at 225rpm in a shaking incubator (Midi shaking incubator SQ-4020, Wolflabs, York, United Kingdom), whilst phage infection took place at its indicated temperature. *S. aureus* was grown at 37°C with constant agitation at 150rpm. All scaled down experiments were conducted in shake flasks using a 20ml volume. For long term storage, bacterial cultures were stored in a 20% glycerol solution at -80°C (Bonilla *et al*, 2016). Phage were stored at 4°C but for long term storage, phage were stored in a 50% glycerol solution at -80°C (Fortier, L. & Moineau 2009). *S. aureus* was grown in brain heart infusion (BHI) media and infected with phage K. For all experiments, *E. coli* was grown using LB media or LB agar. *S. aureus* was grown using BHI media or BHI agar. 0.6% LB agar was used for T4 plaque assays, made from 2g tryptone, 1g yeast, 1.2g bacteriological agar (Thermofisher, Baskingstoke, UK), 2g NaCl per 200ml (Rustad *et a*l, 2018). 0.7% BHI agar was used for phage K plaque assays, made from 7g BHI media, 1.4g bacteriological agar (Thermofisher, Baskingstoke, UK) per 200ml (O'Flaherty *et al*, 2005). Pre-culture of host cells was conducted at 37°C whilst phage infection took place at the indicated temperature.

Propagation, purification with centrifugation and concentration of phage

A single colony of each bacterium from agar plate culture was inoculated in 20ml media and cultured at the respective conditions overnight. Following this, a dilution of the culture was made to

reach an optical density (OD_{600nm}) of 0.05 using a Shimadzu biospec mini spectrophotometer. The culture was grown at 37°C with agitation at 150rpm (*S. aureus*) or 225rpm (*E. coli*) shaking until it reached an (OD_{600nm}) of 0.25 and infected with phage. At the point of harvest, the culture was 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 solution overnight at 4°C. The phage were centrifuged for 1 hour at 4,600g and the supernatant decanted. The pellet was resuspended and stored in LB media for T4 phage or BHI media for phage K.

Enumeration of phage

All experiments were enumerated using the plaque assay. An overnight culture of host bacteria, from a single colony no more than 24 hours old on the respective agar plates, was agitated at 225rpm (*E. coli*) or 150rpm (*S. aureus*) at 37°C was centrifuged at 4,600g for 10 minutes and re-suspended in 3ml fresh media (O'Flaherty *et al*, 2005). The 3ml culture was added to either 5ml 0.6% LB bacteriological agar or 0.7% BHI bacteriological agar for *E. coli* and *S. aureus* respectively (Bonilla *et al*, 2016). The mixture was poured onto fresh LB or BHI agar plate. Appropriate dilutions of the phage were spotted onto the top agar at appropriate serial dilutions. The number of cells at the point of infection, OD_{600nm} 0.25, was used to calculate the phage per input cell whilst the MOI was taken into account to determine the phage output per input phage. Titres achieved are from the purified precipitated post PEG/NaCL purification using a single step purification.

Design of Experiment design

The full factorial experiment was designed using Minitab16. A 4 factor, 3 level design was created equating to 81 experiments for both phage bioprocess design spaces. Table 1 shows the parameters and levels used for each bioprocess. Baseline (or Control) conditions for T4 phage were as follows; MOI 2.5, 225rpm, 3 hours infection, 37°C whilst baseline (control) conditions for phage K were; MOI 1, 8 hours, 150rpm, 37°C, determined from literature review (refer to supplementary data). The baseline acts as a reference point to currently used levels within the literature and acts as a control for changes to the bioprocess to be assessed. Baseline conditions are highlighted in bold. Each of the

varying conditions were run as singular experiments to build the streamlined design space, with enumeration by duplicate plaque assays.

Table 1. A table to show the KPIV and levels used to characterise the bioprocess design space for T4 and phage K in the shake flask model. Baseline (control) conditions shown in emboldened text.

Key Process Input Variable (KPIV)	**T4 Level**	**Phage K Level**
Agitation (RPM)	100, **225**, 400	100, **150**, 200
MOI	1, **2.5**, 10	0.1, **1**, 10
Temperature (°C)	20, 28**, 37**	20, 28, **37**
Time of infection (hours)	1, **3**, 6	4, **8**, 16

Adsorption analysis

All experiments were performed in quadruplicate with each experiment enumerated with duplicate plaque assays. Sacrificial shake flasks were setup with an overnight host culture diluted to 0.05 OD_{600nm} and grown to 0.25 OD_{600nm}. Upon infection of the culture, shake flasks were taken out of the incubator at staggered times every 30s and a 1ml sample was filtered with a 0.22µm filter. The sample was then enumerated as described above.

Bioreactor experimentation

A 5L Biostat B Plus stirred-tank bioreactor (Sartorius, Göttingen, Germany) was used with a 3L working volume, with the greatest titre conditions determined from baseline and small scale factorial experiments of the shake flask model, additional parameters in the bioreactor were dO_2, maintained at 100%, pH maintained at 7.0 and an impellor used for agitation 150rpm (*S. aureus*) and 225rpm (*E. coli*). A single colony of host culture was inoculated in a 1% working volume and grown overnight in a shake flask 37°C. *E. coli* cultures were agitated at 225rpm and *S. aureus* cultures were agitated at 150rpm. The volume was inoculated into the bioreactor and grown to 0.25 OD_{600nm}. The culture was then infected and allowed to grow according to baseline or greatest titre conditions as

determined by shake flask model. Each experiment was completed in triplicate with each experiment enumerated with triplicate plaque assays.

Statistical tests

All statistical analyses were performed using IBM SPSS 23. They included paired two sample t-test and two-way analysis of variance (ANOVA). A p value of <0.05 was considered to be statistically significant.

Results and Discussion

Scaled down optimisation model

The study aimed to improve the batch bioprocess for T4 phage and phage K using a DoE approach. Phage acting against *E. coli* was considered an ideal candidate as it has previously been used in humans who took oral doses to act against *E. coli* K803; whilst Sarker *et al* (2012) applied it to determine how faecal *E. coli* K12 and WG5 counts were affected with no adverse effect noted on the subjects in either study (Bruttin and Brussow, 2005, Denou *et al*, 2009). To ensure the scaled down model and methodology could be developed as an appropriate research tool for use with different phage bioprocesses, it was important to use two exemplar phage bioprocesses with different bacterial hosts; phage K acting against *S. aureus* was chosen due to *S. aureus* also being a target of multiple phage clinical trials because of its antimicrobial resistance threat.

A full 4 factor 3 level DoE design generated 81 runs. The contour plots illustrated Figure 1 show the design space with the zone of greatest output phage titre conditions across the whole experiment shown in the centre of the experiment design (darker green zones indicate higher phage titre). No statistically significant differences were found between titres achieved within each level used for each input variable. The contour plots are therefore useful to show differences in phage titre under combined input variable influence and to check whether the experiment design is appropriate. The design is appropriate as all zones are central within the contour plots, if the zones were not central

and were against the edge of the graph this would suggest that a shift in experimental design was required.

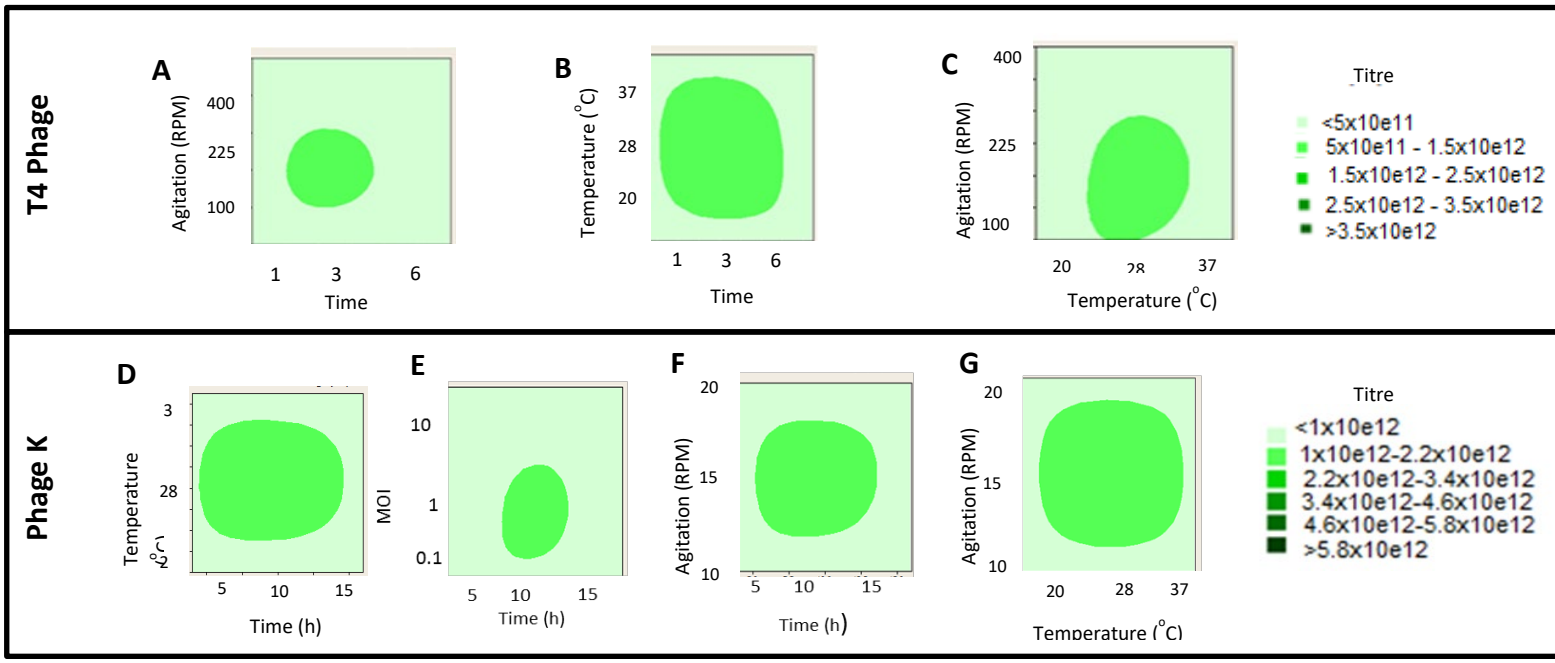

Figure 1. Contour plot analysis of the Scaled down model for T4 and phage K. Contour plots indicating the zones of greatest output phage titre (pfu/ml) for T4 and phage K. T4 graphs A-C, Phage K graphs D-G with respective phage titres in plaque forming units per ml (pfu/ml): (**A**) Time of infection (hours, h) vs agitation during infection (Revolutions per minute, RPM); (**B**) Time of infection vs temperature of infection (°C); (**C**) agitation during infection (RPM) vs temperature of infection (°C); (**D**) temperature of infection vs time of infection; (**E**) Multiplicity of Infection (MOI) vs time of infection; (**F**) agitation during infection vs time of infection; (**G**) temperature of infection vs agitation during infection.

Although no distinct peaks were shown for T4 phage, the contour plots (Figure 1, A-C), showed windows of operation for each parameter where elevated phage titres were achieved. The darker areas within the contour plot (Figure 1) represent conditions where elevated titres, >5 x 10^{11} pfu/ml, can be achieved. However, due to a lack of statistical significance between each levels of the

conditions used, a wide range of levels have been estimated to achieve the elevated titres i.e a distinct peak of optimal input parameters.

A T4 phage titre of >1x 10^{13} pfu/ml was achieved using an MOI of 2.5, 225rpm agitation during infection, 28ºC infection temperature and 3 hours infection time. The T4 baseline process used conditions that are commonly found within the literature, to act as a control. Whilst current T4 processes commonly use a range of MOIs, agitation and times of infection, 2.5, 225rpm and 3 hours was chose to give an overall representitive respectively. However, the greatest difference between currently used conditions and the conditions presented here was the temperature of infection, 37ºC, which achieved an output phage titre of 4.2 x 10^{10} pfu/ml. The greatest output T4 titre was actually 2.2 x10^{13} pfu/ml with slightly different infection temperature conditions of 28ºC which gave around a 500-fold higher titre than at 37 ºC i.e the baseline process which is comparable to the literature (p<0.0001, paired t-test) (4.5 x 10^{10} pfu/ml). There is variation in the literature on achievable levels but this represents greater than 10-fold increase above the highest achievable current levels (Sauvageau & cooper 2010, Bourdin *et al*, 2014, Bonilla *et al*, 2016).

The greatest phage K titre achieved was 6x10^{12} pfu/ml using an MOI of 1, 150rpm agitation during infection, 8 hours infection time at 28ºC temperature, only differing from the phage K baseline (control) conditions in temperature and generating a statistically significant improvement in phage titre (5 x 10^{10} pfu/ml) (p=0.0004, paired t-test). Although this was the greatest output titre, a lower MOI of 0.1, 150rpm agitation during infection, and a lower infection time of 4 hours at 28ºC achieved near identical titres (3.5 x 10^{12} pfu/ml). These conditions were taken forward into further experiments as this output titre was achieved using a lower level of input phage stock and a shorter infection time of 4 hours (compared to 8 hours). As far as the authors are aware this is the first study to determine conditions to maximise phage K titre.

Interaction analysis

The interaction effects plot (Figure 2) shows the mean response for all possible combinations of each input variable and level investigated (described as low, mid or high) for the T4 phage and phage K experiments. Parallel lines within each box indicate no interaction between levels used, however, non-parallel lines that cross indicate statistically significant interactions (p<0.05). Statistically significant interactions were confirmed using a two way ANOVA. The graph shows that 28ºC, MOI 2.5, 225rpm, and 3 hours gave the greatest T4 phage titre whilst 28ºC, MOI 0.1, 150rpm and 8 hours gave the greatest phage K titre.

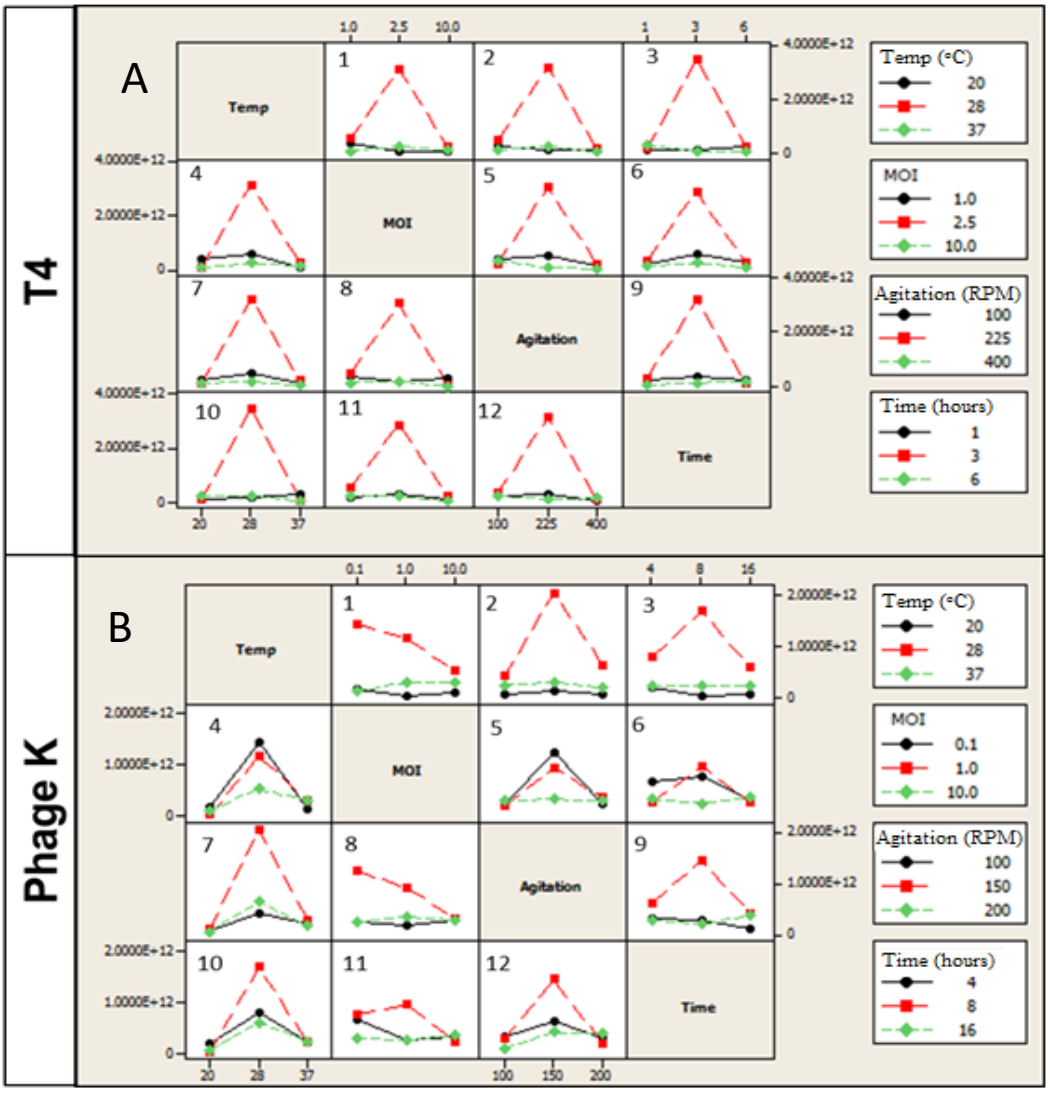

Figure 2. Interaction Effects Plot. The interaction plot shows the mean response for all combinations of input variables and levels investigated for the T4 and phage K scaled down shake flask model. The top plot shows results for T4 and the bottom plot shows results for phage K, within each plot the graphs are numbered showing which input variables are combined as follows: **1** Multiplicity of Infection (MOI) vs. Temperature; **2** Agitation vs. MOI; **3** Time vs. Temperature; **4** Temperature vs. MOI; **5** Agitation vs. MOI; **6** Time vs. MOI; **7** Temperature vs. Agitation; **8** MOI vs. Agitation; **9** Time vs. Agitation; **10** Temperature vs. Time; **11** MOI vs. Time; and **12** Agitation vs. Time. Parallel lines indicate no significant interaction, non-parallel lines that cross indicate statistically significant interactions ($p<0.05$, two-way ANOVA).

T4 interaction analysis:

Temperature: Figure 2a demonstrates that the midpoint for the temperature of infection (28ºC) gives the greatest titre against each of the mid-point levels used for MOI, agitation and time of infection (figure 2a box 1-3). Moreover, against the majority of the high and low levels for the MOI, agitation and time of infection, 28ºC gave a greater mean average T4 titre compared to 20ºC and 37ºC e.g figure 2a graph 1 and 3 at MOI 1, 10 and 6 hours respectively. However, there were some instances where 28ºC did not give the greatest titre. Figure 2a graph 3, shows the greatest mean titre after 1 hour infection is achieved at 37ºC.This may be because a higher infection temperature will favour *E. coli* host growth allowing for a low infection time as the host will replicate faster at 37ºC than at 28ºC or 20ºC, potentially increasing the number of host cells available for phage infection.

MOI: At 20ºC, graph 4 shows an MOI 1 gave the greatest average mean titre. At a low temperature of infection, it may be more beneficial to the titre to use a lower MOI to prevent the host from being infected by more phage i.e a higher MOI will see more host cells infected and if they are unable to replicate due to a low temperature, the overall titre may be reduced. Statistically significant interactions were seen between 28ºC and 20ºC, p=0.019 although there was no significant difference between 37ºC and 28ºC or 20ºC despite the average titre being 3.01e11, 1.59e11 and 1.12e11pfu/ml

respectively. Statistically significant interactions were seen between at an MOI 1 between 20-28ºC and an MOI 10 20-28ºC and 20-37ºC p=0.01, p=0.001 and p=0.001 respectively. The interaction plot shows the influence of temperature of infection on T4 titre irrespective of the MOI or time of infection.

Agitation: The agitation appeared to have the lowest effect on T4 titre. Graphs 7, 8 and 9, Figure 2a, show the mid-point (225rpm) as giving the greatest titre at each mid-point value, however, at the low and high levels, this was not always consistent. Graph 8, Figure 2a, shows that the greatest titre was achieved at 100rpm, MOI 10. Using a lower agitation may prevent optimal mixing within the culture and therefore the phage may not be able to bind to the host and propagate as efficiently as possible. Using a higher MOI may allow more phage to infect the host thus enabling and improving the propagation.

Time: Figure 2a, graph 11 shows a weak interaction between the low and high input variable values i.e near parallel lines, but strong interactions between the mid-point value. This graph shows a weak interaction between each MOI at the low and high infection time (1 and 6 hours respectively) but a strong interaction shown at the midpoint time (3 hours) with all MOIs investigated. Therefore, MOI at the high and low levels has a weak effect on the titre. This trend is also seen in graphs 10 and 12.

Phage K interaction analysis:

Temperature: In addition to the T4 interaction plot, the phage K interaction plot also showed that the temperature of infection played an interesting role on the final phage titre. Figure 2b, graphs 1, 2 and 3 show that 28ºC gave the greatest titre at the high and low levels for all factors investigated. Statistically significant differences were found in the average mean titre between 20-28ºC, 20-37 ºC and 28-37ºC p=0.003, p=0.02 and p=0.014 respectively. Together, this plot adds weight to the argument that a reduced temperature of infection allows for a higher phage titre to be achieved and backs up the results in the contour plot in Figure 1 whilst showing it is the most important factor in phage K propagation.

MOI: Compared to Figure 2a for T4, Figure 2b, graphs 4, 5 and 6 shows far more variance in the MOI and its effect on titre. Graph 5 shows at each of the agitation rates, a different MOI gave the greatest titre. Therefore, this shows that the MOI has little effect on the titre but is worthwhile noting as using a lower MOI will improve efficiency of a bioprocess. Statistically significant interactions were observed at MOI 0.1, 20-37°C, p=0.008 and at 4 hours infection 20-37°C p=0.002.

Agitation: Interestingly, Figure 2b, graphs 7, 8 and 9 show that 150rpm gave the greatest titre at each of the levels used for all conditions. Moreover, 28°C also gave the greatest phage K titre and therefore the interaction analysis shows that the temperature and agitation of infection play the greatest role in phage K propagation. Statistically significant interactions were seen at 100rpm between MOI 0.1-1 and 1-10 p=0.02 and p=0.003 respectively, graph 8.

Time: Similarly to the MOI, the time of infection was shown to be variable with different times of infection giving the greatest titre at the different levels and conditions used. However, at the mid-point of each condition, 8 hours infection always gave the greatest titre, figure 2b graphs 10-12. Given the parallel lines between 4 and 16 hours, a conclusion can be made that 8 hours was the most significant level in respect to time. A significant interaction was seen at 8 hours 100-200rpm and 16 hours 100-200rpm p=0.003 and p=0.01 respectively.

Table 2. The table below shows the most significant interactions seen from the interaction analysis using a two-way ANOVA.

T4	Interaction	P value
	400rpm 28-37 °C	0.000123
	400rpm MOI 1-2.5	0.000072
	1 hour MOI 1-2.5	0.000003
K	20 °C MOI 0.1-1	0.000079
	20 °C MOI 1-10	0.000155
	200rpm MOI 0.1-1	0.000443

	200rpm MOI 1-10	0.000443
	20 °C 150-200rpm	0.000157

The phage K infection process shows far more interactions, between each factor/level used compared to the T4 phage interaction plot. Therefore, each factor and level contributed more to the phage K fermentation process than T4. However, it is known that factors can have different influences on different phage growth parameters (Bourdin *et al*, 2014). Additionally, it was notable to see the effect that temperature had on both bioprocesses and the effect of agitation on the phage K bioprocess with agitation heavily contributed to the phage K titre but had a lesser effect on T4 titre. The results of the experimental design and the interaction analysis were validated by performing nine independent experiment runs of the conditions which gave the greatest titre for T4 phage (MOI 2.5, 225rpm, 28ºC, 3 hours infection). However, phage K used an MOI 0.1, 150rpm, 28ºC, 4 hours infection as a similar titre was achieved when compared to MOI 1, 150rpm, 28ºC, 8 hours infection, $3.5x10^{12} \pm 5x10^{11}$ and $6.5x10^{12} \pm 5x10^{11}$ respectively.

From the nine validation runs T4 phage gave an average harvest titre of $1.87 \times 10^{13} \pm 8.47 \times 10^{12}$ pfu/ml and phage K gave an average harvest titre of $2.41 \times 10^{12} \pm 7.63 \times 10^{11}$ pfu/ml, with no statistical significant difference between any of the validation runs nor the initial experimental scaled down run. A 45% and 55% variation was seen within the phage K and T4 phage validation runs respectively. Due to a lack of distinct peaks in the contour plots, validation of the greatest titre conditions shows they can consistently achieve high phage titres in a reliable manner.

Infection temperature investigation

The results of the interaction analysis showed the temperature during infection was the input variable with greatest influence on output phage titre. To validate this a further study was conducted where infection temperature was altered whilst all other input variables remained constant at the levels determined by the scaled down experiment for maximal output phage titre, T4 phage (MOI 2.5,

225rpm, 3 hours) phage K (MOI 0.1, 150rpm, 4 hours). The experiments were carried out in triplicate with each experiment enumerated with triplicate plaque assays. Additionally, to examine the bioprocesses against each other, normalised values of process output per process input were calculated, these were the phage output per input host cell (host cell number determined at the point of infection) and the phage output per input phage (input phage determined at the point of infection for MOI).

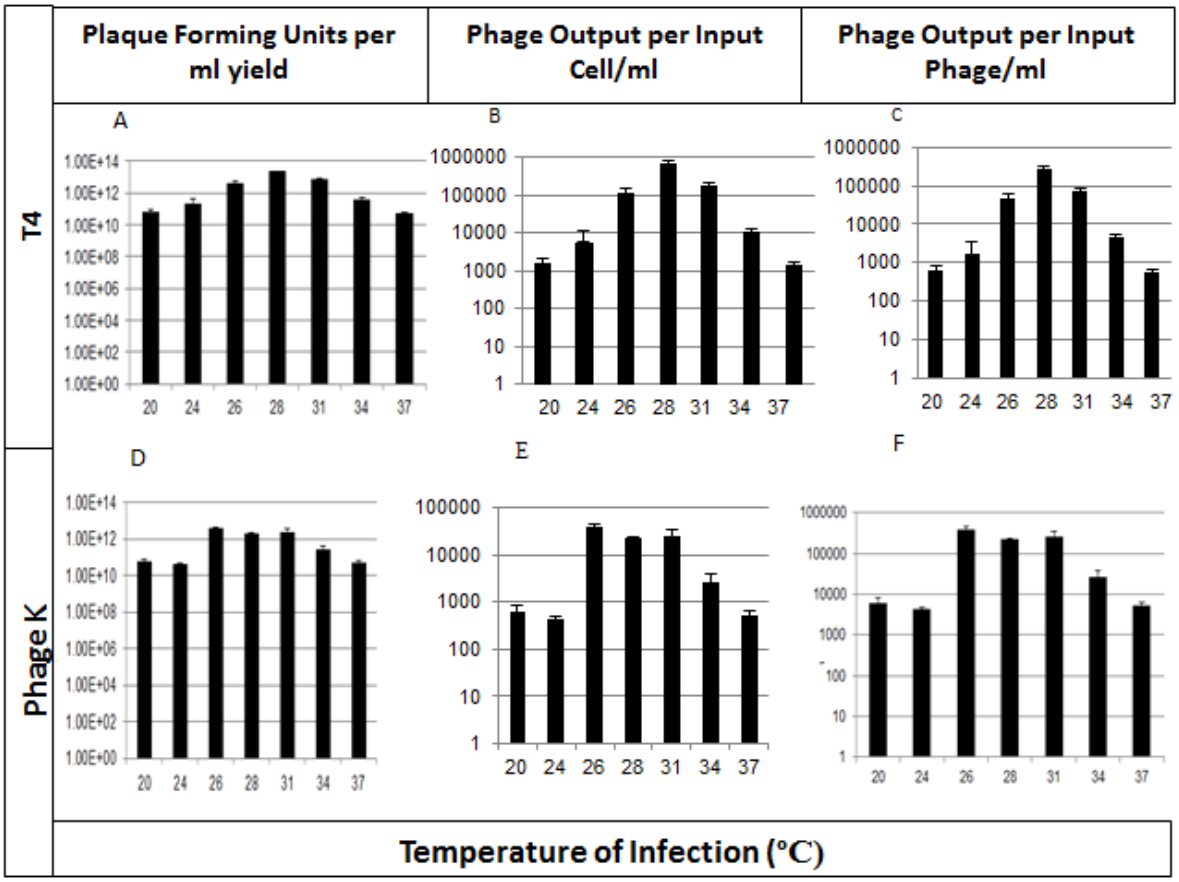

Figure 3. Effects of infection temperature on bacteriophage bioprocess outputs. A range of infection temperatures were investigated (shown on the x-axis for all graphs, A-F) whilst maintaining other input varaibles at previously validated levels for maximal output phage titre. The levels for T4 phage were MOI 2.5, 225rpm agitation and 3 hours infection time. The levels for phage K were MOI 0.1, 150rpm agitation and 4 hours infection time. The experiments were carried out in triplicate and enumerated with triplicate plaque assays. Graphs A-C show T4 phage process outputs, graphs D-F

show phage K process outputs. Column 1 (Graphs A and D) shows output phage titre (PFU/ml), column 2 (Graphs B and E) shows normalised data of number of output phage per number of input host cells (at the point of infection), and column 3 (Graphs C and F) shows normalised data of number of output phage per number of input phage (at the point of infection).

Overall Figure 3 clearly shows a distinct peak of productivity related to infection temperature for both bacteriophage bioprocesses. For the T4 process, the peak sits clearly at 28ºC across all graphs (A-C) with maximal output titre of $2.2x10^{13} \pm 1.2x10^{12}$ pfu/ml. By normalising the data to show output yield vs. input host cells or input phage at the point of infection the improvement to the bioprocess output is more clearly observed. Only the bioprocess with a temperature of infection at 28ºC produced ≥100,000 output phage per single input phage, whereas the bioprocesses with a temperature of infection at either 20ºC or 37ºC were producing <1,000 output phage per single input phage, $p<0.0001$. As Greico *et al* (2012) found only a 10 fold increase in their data, by examining more variables our bioprocess has been able to show almost a 3 order of magnitude increase in T4 phage titre. For the phage K process, there is a window of operation for infection temperature where the greatest output phage titres can be achieved, ranging from 26-31ºC. This result was not unexpected as the contour plot analysis illustrated a wider range of infection temperatures where similarly high output phage titres were achieved in the scaled down model for phage K; this is in contrast to the more defined design space that was achieved for T4. The contour plot analysis predicted infection temperatures between 23-34ºC could achieve $>1x\ 10^{12}$ pfu/ml for phage K, this analysis combined with the scaled down experiment and interaction analyses enabled selection of infection temperature as the input variable with greatest influence on output phage titre. This closer investigation of the infection temperature, after honing in and validating the levels of the other input variables, has demonstrated that titres $>1x\ 10^{12}$ pfu/ml can only be achieved between 26-31ºC (Figure 3, Graph D). Normalised data show that it is only within this infection temperature range that bioprocess productivity achieves levels exceeding 10,000 output phage per single input host cell and exceeding 100,000 output phage per single input phage. Further gains in the output phage per input phage were seen due to the fact that a lower MOI was selected from the scaled down experiment

(from 1 to 0.1). The phage K bioprocesses with lower temperature of infection at either 20ºC or 37ºC were producing <1000 output phage per single input cell or <10,000 output phage per single input phage, in a similar trend to that observed for T4. Statistically significant differences were observed between 28ºC and 20/37ºC between the phage output per input cell $p<0.0001$ for both temperatures and phage output per input phage between 28ºC and 20/37ºC, $p<0.0001$ for both temperatures.

The improvement in phage titre, at a reduced temperature of infection to 28ºC was also observed by Greico *et al*. (2012). Their study showed a 10-fold increase in phage titre could be achieved, when filamentous phage was infected at 28ºC, compared to their best process at 37ºC, however, this is the first study to throughly examine the effect of temperature of infection on phage titre (Greico *et al*, 2012). Hadas *et al* (1997) previously hypothesised that by preventing cell replication, at a lower temperature of infection, the host cells become larger and therefore have more available binding sites for phage. Additionally, Bleckwenn et al (2005) suggested that a temperature reduction may aid in viral protein synthesis and further exploratory work here would be beneficial. Our results show that there is a significant increase in productivity in bacteriophage bioprocessing in relation to the temperature during infection and future work intends to investigate the underpinning biological mechansims for this increase.

Although currently, the gold standard method for phage enumeration is through the plaque assay, normalising the data to phage output per input host cell and phage output per input phage can offer greater insight into the success of the bioprocess. Simply looking at the pfu/ml, does not take into account the MOI which can be highly variable in the literature, with MOI values commonly used anywhere between 1 – 10 (Bourdin *et al* 2014, Bryan *et al* 2016). Whilst these measurements are not widely found within the phage literature, they allow a universal method of enumeration to be used which takes into account the input variables (MOI) whilst also examining the final phage output. Fold expansion and population doubling are used within mammalian cell research and allow authors to easily cross compare their data to others. With phage fermentation research increasing cross comparision this will become more important and phage fermentation research may benefit from a similar method (Kumar *et al*, 2015, Sanz-Ruiz *et al*, 2017, González-Menéndez *et al*, 2018)

Infection kinetics: adsorption and burst size analysis

In order to investigate why the combined input variable analysis led to improved output phage titre an analysis of the kinetics of infection such as phage adsorption and burst size was conducted. The burst size is the number of phage produced per infected host cell and shows the increase in phage titre after a single infection cycle (Golec *et al*, 2014). Earlier adsorption may be occurring with the improved conditions thereby speeding up the infection process and leading to larger burst size upon the first infection cycle. Therefore, the rate of adsorption was determined by examining the number of free phage available every 30 seconds in the culture after the infection (from the point of infection until 5 minutes post-infection). The T4 phage adsorption for the baseline (control) and greatest titre conditions are shown in Figure 4. A statistically significant reduction in the number of free phage was observed at all time points for both T4 and phage K when compared to the baseline process. Therefore showing that by altering the key process input variable parameters, a significant improvement in phage adsorption to host cells can be achieved that contributes to an improved output phage titre. Following this, a statistically significant increase in burst size was also observed for both T4 and phage K when compared to the baseline conditions (Figure 4). There was an average burst size increase of 30% for T4 (p=0.03) and 56% for phage K (p=0.014).

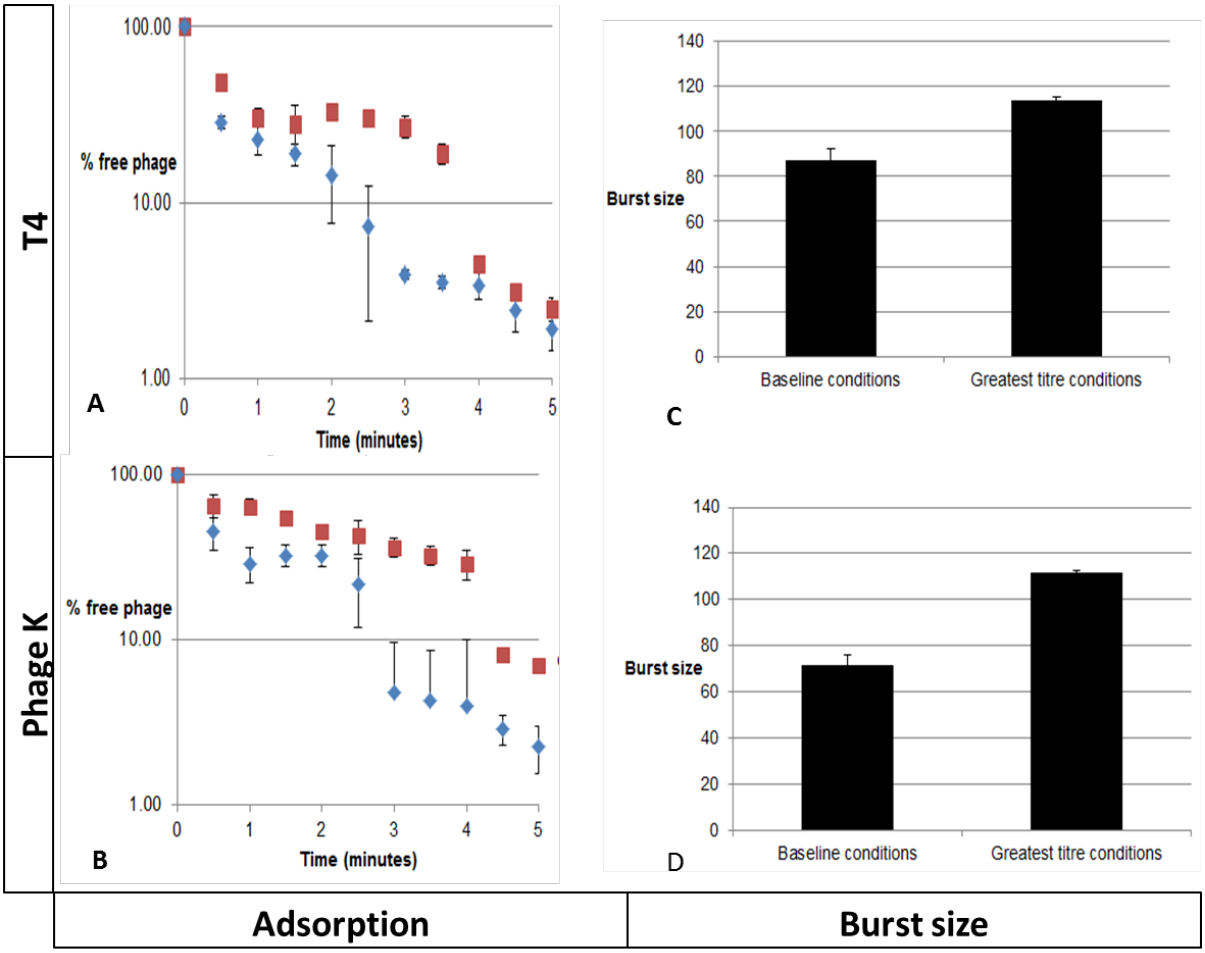

Figure 4. Infection kinetics. The graph shows an analysis in the scaled down model of the adsorption and burst size between the baseline (control) conditions and the full factorial design determined improved input variable conditions. Graphs A and B show the adsorption charts for T4 and phage K with baseline (control) conditions (red squares) and improved output phage titre condition (blue diamonds), depicting the reducing number of free phage available over time after infection. Graphs C and D show increased burst size (the number of phage produced per infected host cell after a single infection cycle) with improved conditions Each experiment was carried out in quadruplicate with individual experiments enumerated by duplicate plaque assays.

Although multiple factors can affect phage adsorption, in this study the temperature of infection significantly improved the adsorption of the phage to its host organism and burst size observed between the conditions, indicating that when infection occurs earlier more phage can be

produced in a shorter time period. Previous studies have shown that over wider ranges of temperatures, the rate of phage adsorption can be significantly affected (Quiberoni *et al*, 1998, Moldovan *et al*, 2007). Whilst Greico *et al* (2012) were able to show an improvement in phage titre at reduced temperature, they were unable to offer an explanation for the improvement in phage titre as infection kinetics analysis was not performed but stated a consideration should be given to the host growth whilst Brown and Bidle (2014) recognised it as a key parameter for viral infection. Wechuck *et al* (2002) showed that viruses are more stable at lower temperatures and when used as vectors can improve yield at lower temperatures. The authors hypothesied that with a lower temperature of infection, the phage DNA may be able to integrate more efficiently and therefore lead to a higher phage yield and therefore cause a bigger burst. Additionally, lowering the temperature of infection may prevent optimal growth of the host and keep the density lower which will be more favourable to phage propagation. Future exploratory work would therefore be hugely advantageous. One study showed that reducing the temperature of infection could upregulate the gene responsible for phage binding and therefore, more phage would be able to infect the host thus producing more phage and may represent an interesting avenue to explore (Tokman et al, 2016).

Whilst the study thus far demonstrated how manipulation of the conditions that contribute to phage infection can significantly improve bioprocess yields and generally improve phage propagation in a streamlined and efficient manner, it was important to translate the bioprocess to a manufacturing scale using a stirred tank bioreactor system. To date, there have been a minimal number of studies examining phage culture in stirred tank systems, however, this is the next logical step in bacteriophage manufacture (Agboluaje & Sauvageau, 2017, Krysiak *et al,* 2018))

Scale up

The translation of shake flask culture to stirred tank bioreactor is not straightforward as there are differences in oxygen transfer rates and mixing due to differences in fluid mechanics, heat transfer and agitation which can alter the bioprocess outputs. To determine whether the effects observed in the

20ml volume shake flask scaled down model were transferable to industrial bioprocessing equipment, the conditions which gave the greatest phage titres were applied in a 3L working volume within a 5L automatically controlled stirred tank bioreactor. To date, a limited body of work exists that examines large scale stirred tank bioreactors for bacteriophage production. However, this translation is welcome as more automated control of cultures and parameter analytics in industrial bioreactors will enable reductions in operator and batch-to-batch related variation. Figure 5 shows the baseline and greatest titre conditions scale up from the scaled down shake flask model (20ml) to 5L bioreactor (3L working volume) for both T4 phage and phage K bioprocesses.

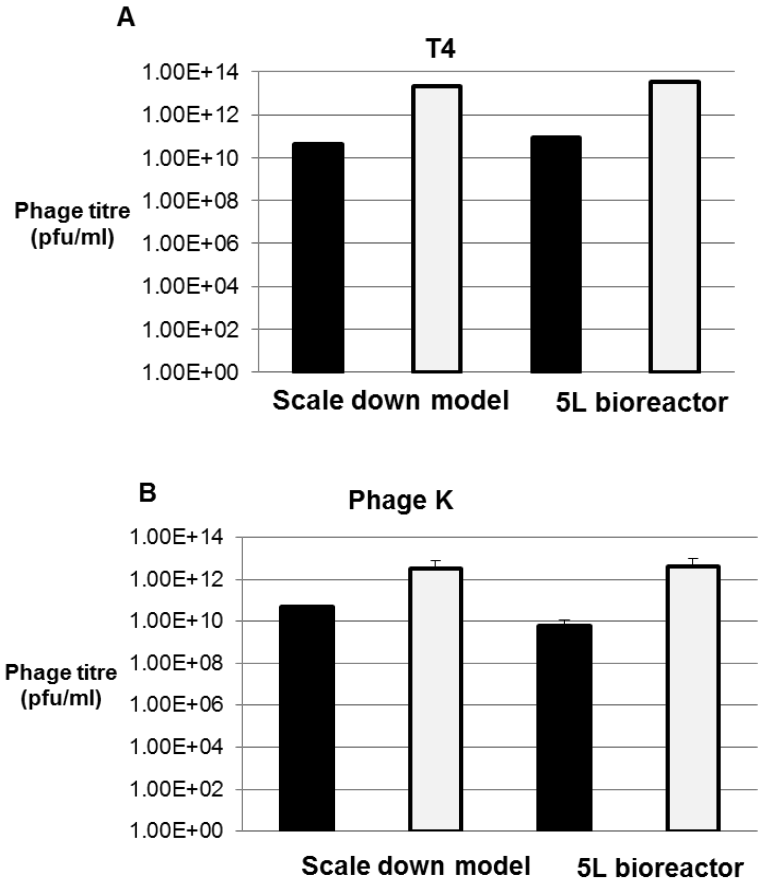

Figure 5. Culture System Output Comparison. The graphs show a comparison of output phage titres between the scaled down culture system (working volume 20ml) and the 5L bioreactor system (working volume 3L). Figure 5A shows the outputs for the T4 phage bioprocess and Graph B shows the phage K bioprocess outputs. Each graph shows a comparison between the baseline phage

process parameters (black bars) and the process parameters that provided significantly improved output phage titres (grey bars) from the scaled down model and the translation to the 5L bioreactor. No statistically significant difference was found between either of the baseline or greatest titre conditions between culture systems for T4 phage using a paired t-test. Each experiment was performed in triplicate with individual experiments enumerated by triplicate plaque assays (bars represent average output titre, error bars represent 1 standard deviation)

The trend of significantly improved output phage titre observed in the scaled down model when compared to the baseline input variable conditions was also observed in the 5L bioreactor for both bioprocesses, thereby confirming that changes in the scaled down model were translated to an industrial scale system. Importantly, within the two culture systems for both phage bioprocesses, there was no statistically significant difference in output phage titre for the greatest titre conditions in 20ml volume or 3L volume, demonstrating that the scaled down model provides a robust starting point for effective process scaling. A significant difference, $p<0.0001$, was found for phage K between the baseline conditions in the two culture systems with the shake flask giving a higher titre. This result shows the potential issues with scaling up a bioprocess and if a similar result has been found for the greatest titre conditions, for either phage, further investigations would be needed.

This is the first study to examine phage yield in a shake flask system and translate the process into a stirred tank bioreactor system. Developing the scaled down model was of critical importance as variable shake flask processes often lead to difficulties in achieving similar yields when the process is moved on into a stirred tank system (Mitchell *et al*, 2000, Garcia-Ochoa & Gomez 2009, Tikhomirova et al, 2018). The research presented here has shown that by narrowing the conditions used and focussing on those that positively influence phage infection, the titre achieved is reliable and validated at small scale and in a scale up system which focuses on larger volumes, automation, and controllable parameters i.e pH and DO_2. Previous studies have shown a greater variance in titre achieved compared to our work as no optimisation of the bioprocess was performed (Bourdin *et al.*, 2014,

Sauvageau & Cooper, 2010). Improving the phage bioprocess in shake flasks allows more rapid experiments to be completed which can then be moved into a stirred tank system. However, further work will investigate differences between optimising for batch bioprocessing and continuous bioprocessing, as well as to investigate the key process inputs and outputs of large scale phage bioprocessing with a view towards maximising the achievable process outputs in a reliable and robust manner.

Conclusion

This study examined the T4 phage and phage K bioprocess using a full factorial design in shake flasks. KPIV were used at a variety of levels to determine their effect on the phage titre. Interestingly, the temperature of infection was shown to have a significant effect on both of the phage titres, whilst the interaction analysis showed the effect of agitation on the phage K bioprocess. This was an interesting result as the phage effect on temperature is something that has been understudied, therefore, this paper explored the temperature of infection within a 4°C range and found nearly an order of magnitude difference between each temperature used. Additionally, the T4 phage bioprocess showed a peak in the phage titre compared to the phage K which showed that there was a window of infection where the greatest titres could be achieved. The curve showed a peak at 28°C which again was consistent with the scaled down experiment for T4 whereas phage K showed a window between 26-31°C. The importance of phage output per input cell/phage was also highlighted and may give a more accurate representation of the bioprocess, by taking into account the MOI. Future phage fermentation studies should focus more heavily on the number of phage per input phage as this gives a more clear understanding of the bioprocess rather than the currently used pfu/ml. Additionally, an investigation into the mechanism of temperature reduction on phage titre improvement would be worthwhile. Examining the cost per phage and phage produced per minute/hour may also be beneficial and gain a further insight into the bioprocess whilst further experiments in the stirred tank bioreactor would be beneficial in order to try and further improve upon the bioprocess.

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