Chapter

Scale-Up and Bioprocessing of Phages

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Abstract

A profusion of new applications for phage technologies has been developed within the last few years, stimulating investigations into the large-scale production of different phages. Applications such as antibiotic replacement, phages as gene therapy vectors, phages as vaccines, diagnostics using filamentous phages and novel optical applications such as the phage laser may need grams to kilogrammes of phage in the future. However, many of the techniques that are used for the growth and purification of bacteriophage at small scale are not transferable to large-scale production facilities of phage in industrial processes. In this chapter, the stages of production that need to be carried out at scale are examined for the efficient large-scale fermentation of the filamentous phage M13 and the *Siphoviridae* phage lambda (λ). A number of parameters are discussed: the multiplicity of infection (MOI) of phage to host cells, the impact of agitation on the initial infection stages, the co-growth with phage rather than static attachment, the use of engineered host cells expressing nuclease, the optimisation of both the quantity and the physiology of the *E. coli* inoculum and phage precipitation methods.

Keywords: phage, PEG precipitation, nuclease, filamentous phage, lambda, phage diagnostics, phage laser, fermenter

1. Introduction

Bacteriophages, often shortened to just phages, are viruses that infect bacteria. Their discovery and characterisation in the early days of bacterial molecular biology has led to certain phages being very well understood in terms of their life cycle, and several phages that infect *Escherichia coli* have become tools in molecular biology techniques such as cloning [1–3]. There has been a resurgence recently in the use of bacteriophages as therapeutics, as vectors for the delivery of vaccines [4], for the killing of pathogenic bacteria as an alternative to antibiotics [5] and for gene therapy to transfer DNA to target human or animal cells [6]. Some of these uses would need the production of many millions of doses of a vaccine, for example, or very large quantities for use as an antibacterial. This has increased demand for investigation into the large-scale production of bacteriophage which would necessitate volumes from hundreds to thousands of litres. The use of phage as biotherapeutics such as vaccines or for gene therapy may be advantageous as phage is considered cheap to manufacture, with large quantities of the product being rapidly produced. But the large-scale production of wild type or genetically modified bacteriophages

for use in the biotherapeutics industry provides significant process and regulatory challenges. Bacteriophages, like any virus, are dependent on a host organism to propagate, in the examples here it is *E. coli*; consequently, the generation of progeny bacteriophage is unequivocally linked to the physiology, molecular biology and growth needs of the host which are important to understand in order to maximise production.

Methods for the production of phages, e.g. λ and M13 bacteriophage, at laboratory production scale have remained unchanged for many years [7]. However, aspects of these protocols are either not practical or unsuitable for large-scale production of phages. Therefore, it is highly desirable to consider early on in the development of phage technologies how any successful bacteriophage therapeutic would be produced at large scale at an industrial level.

One of the problems associated with producing and using λ as a biotherapeutic is the issue of host-derived nucleic acid. The λ lifecycle [8] involves the phage progeny escaping from the host cell by lysis of the bacteriophage host, whereupon the cell contents including high-molecular-weight host chromosomal DNA and RNA are released into the culture supernatant. This significant quantity of host cell-derived nucleic acid can cause important problems for both downstream processing [9] and from a regulatory point of view [10], so reducing the presence of bacterial host nucleic acid in the first stages of the process stream would remove these issues.

M13 is an unusual phage because it does not lyse its host and the entire phage is secreted from the host bacterium through special pores spanning the cell wall [11] although this does make the culture supernatant relatively free of contaminating host cell material, unlike the supernatant in a λ fermentation. In both lytic and secreted phage production the first downstream stage is the concentration of the phage from whatever volume of growth medium was used to grow the infected cells. Filamentous phage such as M13 has a very asymmetric shape with wild-type M13 having a length to width ratio of 138:1 and this extreme asymmetry allows a mild precipitation using polyethylene glycol (PEG) [12].

In this paper we present initial studies into the parameters that need to be manipulated for scaling up fermentation of M13 phage for industrial production.

2. Lambda phage

Lambda (λ) bacteriophage is a temperate phage with a double-stranded (ds) genome of approximately 48 kb [13]. This is encapsulated in an icosahedral capsid (~50 nm in diameter) with a long flexible non-contractile tail (~150 nm in length). The host for λ production is *E. coli* with infection by lambda phage taking place via the maltose binding protein, LamB. Lambda bacteriophage is one of the most intensely studied bacteriophages and has been used for many studies on uncovering basic molecular biology [14] and in biotechnology for phage display of peptides and proteins [15], vaccine [16] and gene transfer and therapy [6].

The large-scale production of genetically modified lambda bacteriophage for use in the biotherapeutics industry provides significant process and regulatory challenges.

One of the problems associated with producing and using lambda bacteriophage as a biotherapeutic is the issue of host-derived nucleic acid. The lambda lifecycle involves the cell lysis of the bacteriophage host, whereupon high-molecular-weight host chromosomal DNA is liberated into the culture supernatant. The presence of large quantities of host cell-derived nucleic acid can cause significant problems during processing as high-molecular-weight chromosomal DNA causes an increase in the cell lysate viscosity [9]. Furthermore, the presence of nucleic acid in the final

product is non-desirable from a regulatory perspective [10], and thus reducing its presence in the first stages of the process stream would alleviate these issues. The lysis of the host *E. coli* cell and the release of intracellular contents (DNA, RNA and proteins) as well as fragments of cell wall will have detrimental effects when processing lambda. For example, intracellular contents can co-precipitate with the phage, can compete for binding sites on chromatography material and can block membranes and chromatography columns. These contaminants need to be taken into consideration when planning a large-scale purification and downstream processing protocol.

3. M13: a filamentous bacteriophage

M13 is an unusual phage as it has a filamentous structure of 900 nm in length and 6.5 nm in width. It is a member of a small group of closely related phages including F1 and Fd [17] that infect *E. coli*. The genome is a single-stranded circular DNA molecule, and the length of the phage (but not its width) is simply determined by the size of the page genome. Short phage particles can be made using plasmids that contain just the replication origin and packaging signals, and phage particles longer than the wild type can be made by inserting DNA sequences into the phage genome. It was thought that the very long but thin shape of M13 and other filamentous phages would increase their shear sensitivity in the various kinds of industrial-scale processing equipment of pumps, continuous centrifuges and membrane filters. This was seen not to be the case [18] which is highly advantageous for large-scale downstream processing of this and other filamentous phages. Filamentous phages have a rather special property in that they do not lyse their host, but set up a permanently infected state, and new, progeny phage is extruded through special structures in the cell wall. Derivatives of M13 phage were extensively used in the early years of DNA sequencing by the Sanger method [19] and in the techniques of site-directed mutagenesis [20] and phage display [21] for the maturation of recombinant antibodies [22].

The unusual mode of growth of filamentous phage by secretion from the host without lysis has considerable advantages for these molecular biology methods because the phage in the supernatant of growing cells is relatively free of any cellular contaminants [23] such as intracellular proteins, genomic DNA and RNA. This makes the purification of filamentous phage a relatively simple matter with many fewer contaminants than phage λ cultures.

4. Multiplicity of infection (MOI)

The multiplicity of infection or MOI is the number of phage particles added per host cell to initiate infection and thus production. The methods developed for the uses of phage λ and M13 at small scale or a few mL generally tend to use a high MOI of 5–10 or more. The MOI is important for scale-up as it defines how large the culture that provides the phage for the scale-up needs to be. It is neither desirable nor sensible to have to grow a fermenter of phage to inoculate a slightly larger fermenter in the final preparation. Also with a large MOI of just under 10, we reach the point where there will be enough phage for every cell to be infected, and at that MOI we can only expect a single burst of phage particles for lytic phage and therefore only a small increase in the number of phage added. With an MOI of 1, only 63% of cells will be infected by one or more phages because there is a Poisson distribution of MOI to infected cells [24]. But some phages have many binding sites per cell, e.g.

T4 has 10^5 molecules of OmpC to bind to [25], while M13 only has approximately 3 [26], so the kinetics of phage finding and attaching to bacteria and forming a productive infection are quite complex. There are 30,000 LamB proteins in the outer membrane which is the initial receptor for λ but many fewer copies of the mannose receptor, ManY, in the inner membrane which is where the DNA of λ crosses the inner membrane [27, 28]. For M13 the receptor is the tip of the F plasmid's transfer pilus, and there are usually one or two F pili per cell. But once M13 has established its replication inside an *E. coli* host, the cell is then permanently infected and will continue to secrete phage from these intact cells.

Under ideal conditions the burst size of λ phage particles is 170 +/− 10 which takes 51 min [29], and during this time uninfected cells will still be growing and dividing, providing new hosts for the phage that are released. To get repeated rounds of replication, the ideal cell numbers and MOI for each phage are different and take into account cell division rates, numbers of receptors, the choice between a lytic and lysogenic cycle in phages where those can take place, the burst size and rate of replication and maturation of the phage. This complex interaction of several parameters means that it is difficult to say a priori what the combination of cells, phage and time of addition is the most appropriate for a given size of growth chamber.

Figure 1 shows the relationship between the host *E. coli* and MOI of M13.

The graph of MOI and final phage production in **Figure 1** shows that from a 10^6 range of MOI added at the start of growth in **Figure 1**, all three cultures reach the same final M13 phage titre. It just takes slightly longer for the lower MOI cultures to reach the final of approx. 5×10^{11} phage per mL. This has important consequences for scale-up of M13 production. For example, if a large fermenter of, say, 100 L were needed and the MOI of 50:1 was needed, we would need to have 500 mL of the equivalent inoculum used here. The information from **Figure 1** shows that we can use just 0.5 mL of the same titre inoculum or much less, e.g. down to a few microlitres. For convenience it is best to inoculate a fermenter with enough in terms of volume that will reach the medium in the fermenter, so a few millilitres of a phage dilution are all that is needed. This means that one phage stock can be used for multiple fermentation runs.

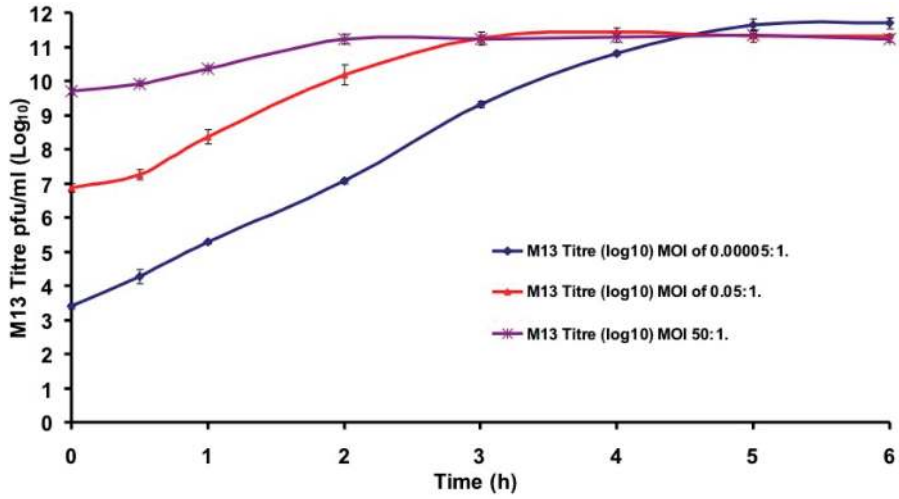

Figure 1.
Titre of M13 produced from different MOI on E. coli TOP10 F'. M13 phage was prepared from a 400 mL culture of E. coli TOP10 F' and precipitated with PEG 6000 at a final concentration of 3.3% and 330 mM NaCl. The precipitated phage was centrifuged at 14,000 ×g for 10 min at 4°C and resuspended in 8 mL of 10 mM Tris.HCl pH 7.5 and filtered through a 0.22 μm filter. The stock of M13 was approximately 1×10^{12} pfu/mL. E. coli TOP10 F' was grown in 400 mL of Nutrient Broth No. 2 (Oxoid) and a 40-mL inoculum in 2-L shake flask at 37°C. Around 2 mL of M13 was added at the appropriate dilutions to achieve the three different MOI of 0.00005:1, 0.05:1 and 50:1. Each point is the average of three flasks.

5. Initial phage binding and infection in fermenters

The standard way of initiating infection of λ or M13 is to mix the phage and cells and allow a static incubation for usually 5–15 min for phage attachment to the phage binding target on the cell surface. The phage/cell mixture is then added to broth if liquid growth is desired or to 3 mL of soft agar and poured onto an agar plate if plaques are wanted. This static incubation is in the recipes for all phages being handled at the lab scale and probably came about because researchers thought it would maximise the attachment of phage to their host. However, in a fermenter it is not sensible to turn off the stirrer and let the cells sit for 15 min while phage attachment takes place. We tested what would happen if M13 phage were simply added directly to shaking cultures of *E. coli* JM107 without stopping the shaking and comparing this with a static attachment/incubation of 30 min.

Figure 2 shows this experiment with three different inocula of M13 phage giving MOI ranging over a 10^6 range.

It is clear from **Figure 2** that the static incubation is not needed for the initial attachment of M13 to sensitive *E. coli*, and so the required dose of M13 phage can be directly added to a fermenter with the *E. coli* host already growing with the impeller stirring. The culture which will be stirred at a high rate *does not* need to be stopped and left to go static for 15–30 min. This would compromise the growth of the cells in a large fermenter, and so our findings give a positive help for the scale-up of phage and the way one can run a large fermenter where procedures have to be different from what is done at small scale in the molecular biology lab.

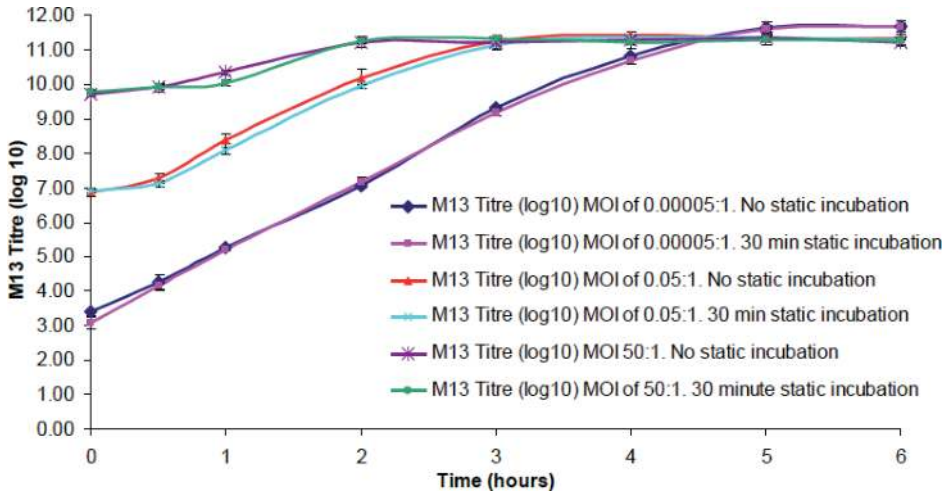

Figure 2.
Static versus shaking incubation of M13 with E. coli TOP10 F'. The conditions and culture quantities were identical to those shown in ***Figure 1*** *with the addition of three conditions at each MOI where the M13 phage was added and left for 30 min static incubation. These cultures for the static incubation were then grown with shaking at 200 rpm in a 37°C orbital incubator. Each point is the average of three flasks.*

6. Nuclease-producing *E. coli*

The above sections on MOI and removing the necessity for a static attachment of phage were shown with M13 as examples. The M13 phage does not lyse its host, so the culture medium after infection is uncontaminated by the bulk of the host cellular contents and largely contains just the filamentous M13 bacteriophage particles. We have examined the supernatants from M13-infected cultures and determined the levels of host cell contaminants of DNA and protein [23] which are quite low

compared to the large amount of cellular DNA, RNA and protein released by lytic phage. A phage such as λ is a representative of lytic phage which is the majority of the types of phages used in therapy and biological control for the replacement of antibiotics. At each cycle of replication and release of the new phage particles, the host is lysed, and the cell contents are released into the medium along with insoluble debris from the cell wall and membrane. This leads to problems in subsequent downstream purification due to the large amount of different cellular molecules competing in the subsequent downstream processing steps.

The host RNA and DNA represent major contaminants that need to be removed especially for gene therapy applications. The release of host cell DNA also increases the viscosity of the medium, and this has an adverse effect on clarification by centrifugation and membrane concentration due to blocking of the membranes. For lab-scale molecular biology work, it is normal to add the enzymes pancreatic RNAseI and pancreatic DNAseI from bovine pancreas preparations. With the advent of bovine spongiform encephalopathy (BSE) which peaked in the 1990s, the addition of any bovine or animal proteins into the growth or purification train of material destined for human therapy was banned. These regulatory restrictions removed the ability to use these cheap nucleic acid-degrading enzymes, and the substitutes from bacterial sources were much more expensive. A strategy to overcome this problem was developed by us, and this was to express a broad-spectrum nuclease in the periplasm of *E. coli* which would be released as cells were lysed [30, 31]. The enzyme Staphylococcal nuclease has been extensively characterised and used from the 1960s onwards [32] and can degrade both DNA and RNA. The expression of this in *E. coli* where it is secreted into the periplasm does not affect the growth of *E. coli* because the enzyme cannot get access to its substrate while the cell is growing normally. When the cells are lysed by a bacteriophage such as λ or by homogenisation, the nuclease can gain access to the released DNA and RNA and degrade them. This 'cell engineering' approach to assisting bioprocessing was developed at UCL and has been shown to give considerable gains in the centrifugation steps and other downstream purification steps in bioprocessing of proteins such as Fab fragments [33–35].

We sought to apply this cell engineering strategy for the production of λ phage and to help solve the problem of the large amount of cellular contaminants released into the media when λ phage cultures need to be harvested and processed.

E. coli JM107 [3] was grown in 2 L fermenters with either JM107 or JM107 containing the plasmid pMMBompnucB which is a broad host-range plasmid vector based on an Inc. Q plasmid RSF1010 and contains the Staphylococcal nuclease which has been altered by the addition of the *E. coli* ompA signal sequence for secretion [31]. **Figure 3** shows the growth of the two hosts with no added λ phage and the same hosts with 8×10^{10} λ phage particles added after 2 hours when the OD_{600} had reached 10.

The addition of λ to the fermenter used the strategy that we had developed where a low MOI is used and without a static initial incubation of the host cells and the phage. In this way we can add the phage directly to a growing fermenter of host with the impeller (single shaft with three top-driven, equally spaced, six-bladed turbines) and four diametrically opposed baffles in the fermenter, running throughout. The growth profiles of the two uninfected cultures show no difference in their growth profiles which means that the presence of the expressed nuclease enzyme in the periplasm has no effect on growth rate or final OD. In the two cultures with added λ phage, the OD drop is the same for both hosts showing that λ replication and cell lysis are the same in both.

The production of λ was monitored throughout growth and is shown in **Figure 4**.

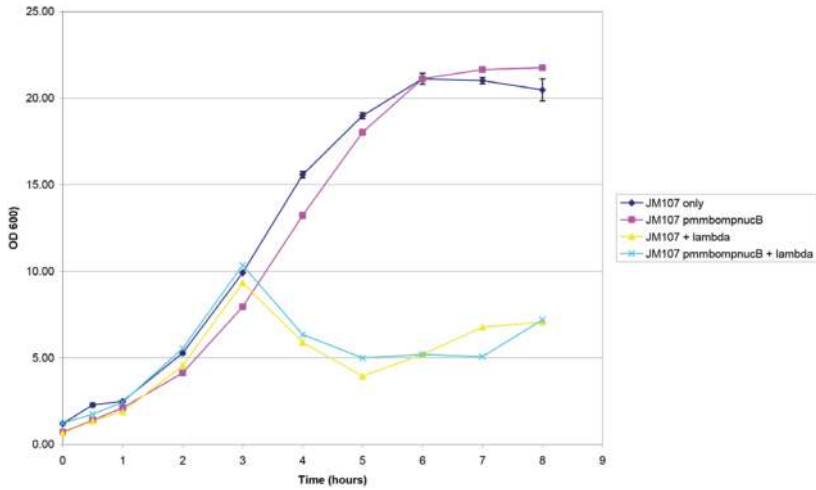

Figure 3.
Growth of E. coli JM107 and λ production with and without a nuclease-expressing plasmid. E. coli JM107 containing pMMBompnucB and with no plasmid was grown in 2 L (working volume 1.5 L) LH 210 series fermenter (Bioprocess Engineering Services, Charing, Kent, UK) with 150 mL of E. coli inoculum and a final working volume of 1.5 L in phage media containing 100 μg/mL ampicillin and 20 μg/mL IPTG. λ phage particles were added at 2 hours to give an MOI of 0.05 (4 mL of 2 × 10^{10} pfu/mL). Impeller stirring continued throughout the growth and addition of phage, and the OD_{600} was monitored.

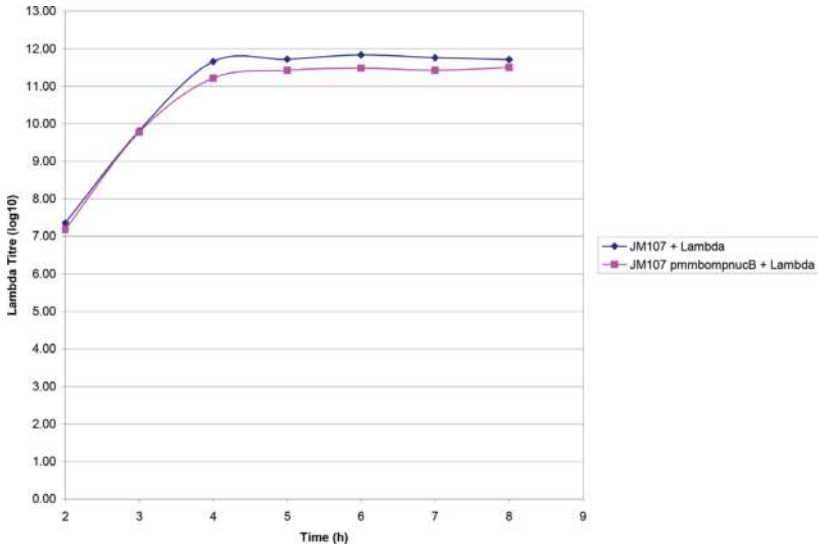

Figure 4.
Production of λ phage in 2 L fermenters with and without periplasmic nuclease expression. λ phage was added at 2 hours in ***Figure 3****, and the graph here shows λ phage amounts from the 2 hours onwards. Samples from the fermenters shown in* ***Figure 3*** *were diluted in phage buffer and titred on E. coli JM107 using soft top agar and plaque counting.*

Both the nuclease and the non-nuclease-expressing *E. coli* JM107 produced the same 5-log increase in phage particles with the same time profile. The efficacy of the nuclease in the removal of the host nucleic acid was assessed by the electrophoresis of samples from each time point on agarose gels and the visualisation of the released nucleic acid (both DNA and RNA). **Figure 5** shows the complete degradation and removal of the released host genomic DNA and host RNA in the strain of *E. coli* expressing the periplasmic nuclease.

The presence of the expressed periplasmic nuclease is apparent from the difference in the samples in **Figure 5B** compared to the samples from the same time points in the fermenter with no expression plasmid for the Staphylococcal nuclease.

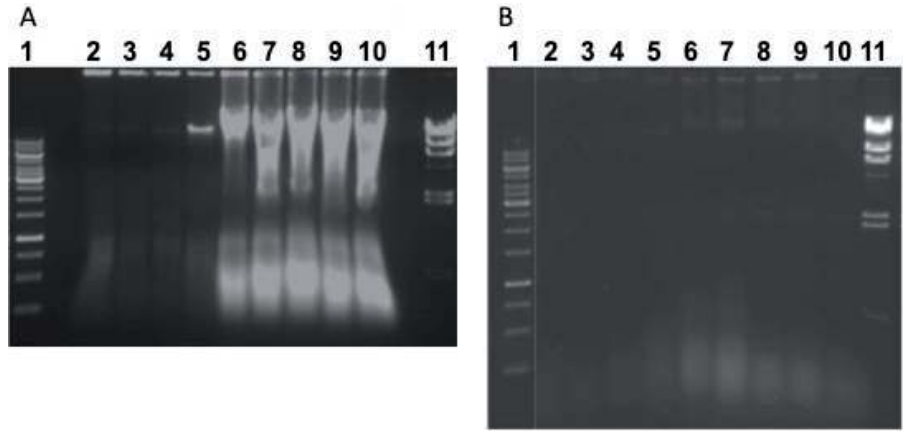

Figure 5.
*Degradation of host nucleic acid from l infected E. coli JM107 and JM107 containing pMMBompnucB. (A) Agarose gel with samples from fermenter in **Figure 3** growing E. coli JM107 with λ. 1, 1 kb ladder; 2, 0 h; 3, 1 h; 4, 2 h; 5, 3 h; 6, 4 h; 7, 5 h; 8, 6 h; 9, 7 h; 10, 8 h; 11 λ HindIII ladder. (B) Agarose gel with samples from fermenter in **Figure 3** growing E. coli JM107 containing pMMBompnucB infected with λ. 1, 1 kb ladder; 2, 0 h; 3, 1 h; 4, 2 h; 5, 3 h; 6, 4 h; 7, 5 h; 8, 6 h; 9, 7 h; 10, 8 h; 11 λ HindIII ladder. The same volume of sample from each fermenter time point was loaded onto each lane.*

Almost all of the released host genomic DNA and the large majority of the RNA has been degraded in the culture that expresses the nuclease. **Figure 4** shows that the production of λ phage particles is identical in both fermenters, and the presence of the nuclease does not impinge on λ production and leads to a removal of the majority of the nucleic acid that would normally need the addition of bovine pancreatic DNAseI and RNAseA or more expensive bacterial equivalents such as Benzonase™ [36] to reduce the amount of nucleic acids. This cell engineering approach means that no animal-derived enzymes need be added, no costly commercial bacterial enzymes need be added and the engineered cells provide their own nuclease which degrades the nucleic acid in situ, so no additional time for incubation of any added enzyme is needed. Therefore a saving of both time and money is achieved via cell engineering for bioprocessing.

7. Precipitation of M13

Bacteriophages produced at any scale need to be concentrated by some method after the growth and production have taken place. The properties of phages allow some precipitation methods that are milder than conditions needed to precipitate host soluble protein or nucleic acid. Phages are large multicomponent entities usually many hundreds of times larger than a medium-sized soluble protein. Their asymmetric shape also enables mild precipitation methods. Polyethylene glycol (PEG) precipitation is a mild method of precipitating biological material and is very efficacious in the precipitation of large asymmetric material such as DNA or macromolecular assemblies, e.g. virus-like particles such as phages [37, 38]. The larger and more asymmetric the particle, the lower the amount of PEG is needed to precipitate the particle and leave behind other smaller soluble materials. An exploration of different average molecular weights of PEG from 600 to 20,000 for M13 precipitation showed that PEG 6000 and PEG 8000 combined the best properties of precipitation at low % concentration with lower viscosities than PEG 12,000 and PEG 20,000 [23] and 2% PEG 6000 with 330 mM NaCl gave >95% precipitation of M13. The relationship between PEG and NaCl is shown in **Figure 6** where the increasing PEG molecular weight and PEG concentration with % of M13 recovered were investigated.

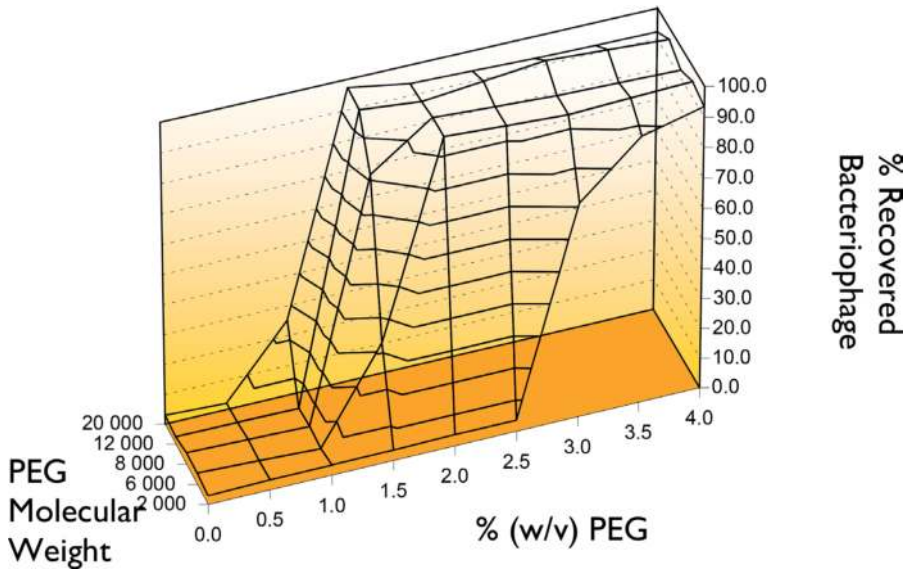

Figure 6.
The relationship between M13 recovery and PEG molecular weight and percentage PEG. All precipitations were carried out at 10 mL scale with additions of PEG to the final % concentration shown in the diagram with 4×10^{12} CsCl purified M13 and 330 mM NaCl. The mixtures were incubated on ice for 1 hour and centrifuged for 10 min at 8000 ×g at 4°C.

The diagram in **Figure 6** shows the relationship between increasing chain length/molecular weight of PEG, the % of PEG and the amount of M13 phage recovered. Higher-molecular-weight PEG preparations can be used at low final percentages, but there is significant increase in the viscosity of high-molecular-weight PEG solutions in the stock solutions needed to add to the phage-containing solution, making PEG 20,000 very difficult to use. PEG 6000 and PEG 8000 achieve virtually the same precipitation profile as PEG 12,000 and the lower viscosity of stock solutions of PEG 6000 make this chain length the best for precipitation with low viscosity. A concentration of 2% w/v PEG 6000 is ideal with 330 mM NaCl [23]. It was discovered that the nutrient media commonly used for growth such as NB2 contains sufficient salt (Na, K and NH_3 ions) that the added NaCl can be reduced to only 135 mM which would make a saving in materials and disposal costs in large-scale M13 phage precipitation.

8. Conclusion

The strategies for the scale-up of phage growth and primary downstream purification are still in their infancy, but we have shown that there are significant gains to be made from the work described here. The amount of phage that needs to be added to large-scale growth volumes can be reduced by several orders of magnitude from what is common at the lab scale. Phage can be added directly to fermenters where rapidly stirring impellers are needed to maintain aeration and correct physiology of the host with no cessation of the stirring, and the phage will find their host and attach with no difficulty. The use of cells engineered to produce their own broad-spectrum periplasmic nuclease gives significant gains in the destruction of host DNA and RNA release on lysis, and this prevents the contamination of the phage with nucleic acid when the phages are concentrated by precipitation. The gentle precipitation method of using low concentrations of PEG can then be used to give relatively pure preparations of phage in one step. These methods can be used together and will

allow the large-scale uses of phage in the future in medical and clinical applications and then beyond into biotechnological applications such as the uses of filamentous phage in electronics like phage batteries [39] and the phage laser [40].

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Conflict of interest

There are no conflicts of interest.

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