Investigation into efficient bacteriophage production for use against enterotoxigenic *Escherichia coli*.

by

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Dedication

My work is dedicated to my mom, Karen, and my husband, Mathieu; your love and support are unparalleled and my life is forever blessed to have you two be a part of it. Thank you for being with me on my education journey.

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Abstract

Piglets are highly susceptible to infection by enterotoxigenic *Escherichia coli* (ETEC), resulting in severe cases of diarrhea. However, due to the growing resistance to antibiotics, there is a demand for bacteriophages (phages) to serve as an alternative treatment option against ETEC. Unfortunately, no defined method to produce high titer ETEC phages on a large scale has been published, limiting phage usefulness as a therapeutic against this disease. Because of this, the present work focused on optimizing conditions to produce high titers of an ETEC phage, designated 'Phage75', by exploring several variables. Variables considered for optimization were multiplicity of infection (MOI), media composition, temperature, harvest time, and alternative host. To screen variables, a high-throughput assay was used to monitor optical density at 600 nm (OD_{600}) of host bacteria since a decrease in OD_{600} is indicative of cell lysis and therefore phage production. Conditions from these assays were carried out in shake flasks at increasing volumes to determine phage titer, assess scalability, and help define an efficient production strategy. These studies revealed that population-wide lysis and peak titer coincide with each other and that extending production does not yield more phage. Furthermore, comparable titers in the range of 9.45-10.10 $\log_{10}$(PFU/mL) were achieved at different volumes in shake flasks as well as in a CellMaker bioreactor. This suggests the proposed method to produce Phage75 is a robust process and may possibly provide insight to produce other phages in the future. The optimized method for Phage75 is: 4% infection load, 10^{-4} MOI, complex media (either LB-Lennox or Select APS) with no ion supplementation, 37°C incubation temperature, and 4-hour harvest time. Additionally, based on alternative hosts tested, it is advised to produce Phage75 on the host it was isolated on to avoid a sacrifice in titer. For phages to become a viable treatment option against bacterial disease, efficient production strategies are needed. To achieve

this, investigations into different growth conditions and their impacts on phage production are necessary and of the utmost importance.

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List of Abbreviations

APMs – antimicrobial peptides

APS – select alternative protein source

BHI – blood heart infusion

Ca^{2+} – calcium ion

$CaCl_2$ – calcium chloride

cAMP – cyclic adenosine monophosphate

CER – carbon dioxide evolution rate

CFTR – cystic fibrosis transmembrane conductance regulator

CFU – colony forming unit

cGMP – cyclic guanine monophosphate

CPM – cycles per minute

CT – *Vibrio cholerae* heat-labile toxin

dCER – first derivative of CER

EAST1 – enteroaggregative heat-stable toxin 1

EM – electron microscopy

EOP – efficiency of plating

FDA – food and drug administration

F41/F4 – fimbriae type 41 and 4

GC-C – guanylate cyclase C

HCl – hydrogen chloride

IL – infection load

Kbp – kilo base pairs

K_2HPO_4 – dipotassium phosphate

LB – Luria Bertani (Lennox)

Lpm – liters per minute

LT – heat-labile toxin

$MgSO_4$ – magnesium sulfate

MOI – multiplicity of infection

NaCl – sodium chloride

Na_2SO_4 – sodium sulfate

OD_{600} – optical density at 600 nm

Opt. M9 – Optimized M9 minimal

PFU – plaque forming unit

Rpm – rotations per minute

SM – saline magnesium

ST – heat-stable toxin

TJs – tight junctions

Tris – tris (hydroxymethyl) aminomethane

TSB – tryptic soy broth

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CHAPTER 1.0: LITERATURE REVIEW

1.1 Enterotoxigenic *Escherichia coli*

1.1.1 Introduction to enterotoxigenic *Escherichia coli*

Enterotoxigenic *Escherichia coli* (ETEC) is a gram-negative bacilli, facultative anaerobe, and an enteric pathogen of humans and animals (Fairbrother & Nadeau, 2019; Nagy & Fekete, 2005). Many strains of ETEC exist and are distinguished based on their different combinations of virulence factors referred to as, 'virotype' (Fairbrother & Nadeau, 2019; Luppi, 2017). For ETEC to cause disease in mammalian hosts, specific interactions with enterocyte receptors must be made to allow sufficient colonization (Fairbrother & Nadeau, 2019; Nagy & Fekete, 2005). This required level of specificity is often not a problem due to different fimbriae types associated with ETEC, and yet animals such as horses, poultry, and rabbits are not prone to ETEC infections (Dubreuil et al., 2016; Gyles & Fairbrother, 2010). However, it is common for ETEC to cause disease within humans and livestock such as pigs, cows, and sheep (Dubreuil et al., 2016).

1.1.2 ETEC virulence factors

1.1.2.1 Adhesions

ETEC adhesion to enterocytes is achieved with the use of fimbriae that cover the surface of bacterial cells. Fimbriae allow ETEC to successfully adhere to and colonize the intestinal tract without damaging the lining, reaching numbers of 10^9 cells per gram intestine (Dubreuil et al., 2016; Nagy & Fekete, 1999). While ETEC can cause infections in both humans and animals, different fimbriae are required based on available intestinal receptors, highlighting a key structural difference among virotypes. For example, fimbriae families F2 and F3 specifically

recognize and bind enterocytes in humans, where as fimbriae families F4, F5, F6, F17, F18, or F41 are required for the same action in pigs and calves (Nagy & Fekete, 2005). Because of this level of specificity in their attachment, ETEC is not considered to be zoonotic (Nagy & Fekete, 2005; Qadri et al., 2005).

1.1.2.2 Enterotoxins

Once a high population of ETEC is established in the intestine, several different enterotoxins are released, broadly classified as either heat-stable (ST) and heat-labile (LT). The ST enterotoxins are further divided into subgroups STa and STb, as well as enteroaggregative heat-stable toxin 1 (EAST1) (Fairbrother & Nadeau, 2019). These enterotoxins individually or in combination wreak havoc on the host by disrupting the fluid and electrolyte balance, resulting in diarrheal disease.

1.1.2.2.1 Heat-labile toxin mechanism of action

ETEC LT is structurally and mechanistically similar to *Vibrio cholerae* heat-labile toxin (CT) (Nagy & Fekete, 1999). LT and CT bind to the surface of cells via the GM1 ganglioside receptor, induce endocytosis, and become cleaved into an activated form by enzymes in the endoplasmic reticulum. This toxin fragment goes on to activate the enzyme adenylate cyclase, leading to the overproduction of cyclic adenosine monophosphate (cAMP). The increased amount of cAMP starts a series of events that include the activation and opening of the cystic fibrosis transmembrane conductance regulator (CFTR) causing an influx of chloride and bicarbonate ions into the intestine. This consequently causes an influx of water into the intestine in an attempt to restore homeostasis, though it is a futile effort since the mechanism of LT is irreversible. Additionally, LT is thought to compromise the tight junctions (TJs) of enterocytes causing further leakage into the intestine (Dubreuil et al., 2016).

Antimicrobial peptides (AMPs) are made by various cell types such as neutrophils, leukocytes, macrophages, and intestinal Paneth cells, and are part of the innate immune system (Q.-Y. Zhang et al., 2021). AMPs are effective against invading bacterial pathogens in many ways including compromising their membrane integrity or inhibiting their protein and nucleic acid synthesis (Huan et al., 2020). However, toxins LT and CT can supress the expression of AMPs in the intestine (Chakraborty et al., 2008), taking away this first line of defence and possibly contribute to the mechanistic irreversibility these toxins have.

1.1.2.2.2 Heat-stable toxin mechanism of action

1.1.2.2.2.1 STa and STb enterotoxins

STa enterotoxin is 50% homologous to guanylin hormone, allowing it to bind transmembrane protein guanylate cyclase C (GC-C) and activate it (Dubreuil et al., 2016; Gyles & Fairbrother, 2010). This activation leads to the overproduction of cyclic guanosine monophosphate (cGMP) which goes on to activate and open the CFTR. The effect of this is influx of chloride and bicarbonate ions into the intestine, as well as water, in attempt to restore fluid/electrolyte balance. Additionally, STa prevents sodium reabsorption in the intestine (Gyles & Fairbrother, 2010), and also negatively impact the integrity of the TJs. This mechanism is much like that of LT; however in this case, the effects of STa are reversible (Dubreuil et al., 2016).

STb enterotoxin presents similar cellular consequences as STa; however, it binds to sulfadite, a glycosphingolipid commonly on enterocytes, and results in increased Ca^{2+} uptake. Increased Ca^{2+} concentration within cells activates protein kinase C which goes on to activate CFTR, again, leading to ion and fluid loss (Dubreuil et al., 2016; Gyles & Fairbrother, 2010).

There is also evidence suggesting STb may increase the permeability of intestinal cells, contributing to these water and ion losses (Dubreuil et al., 2016).

1.1.2.2.2.2 EAST1

EAST1 shares structural similarity with guanylin and is thought to follow a similar mode of action as STa and bind the GC-C receptor to elicit disastrous effects within a host (Dubreuil et al., 2016). However, despite extensive intestinal colonization, *E. coli* mutants containing EAST1 as the sole enterotoxin do not result in increased intracellular cGMP nor disease when inoculated in 5-day old piglets. Furthermore, ETEC strains containing EAST1 and either STa or LT enterotoxin do not yield higher concentrations of cyclic compounds in either human or porcine cell lines, suggesting the enterotoxins are not synergistic (Ruan et al., 2012). Interestingly enough, a study has revealed EAST1 to be more commonly found in bacterial samples from healthy pigs than those suffering from an ETEC infection (Zajacova et al., 2012). In the same study, EAST1 was seen to be more prevalent in strains containing LT and not STa, suggesting some relation that has yet to be determined.

1.1.3 Resulting disease

1.1.3.1 Humans

ETEC commonly causes 'Traveler's Diarrhea' in those travelling to developing countries in South America, Africa, Asia, and the Middle-East, due to contaminated water and food (Adler et al., 2022; Qadri et al., 2005). Consuming ETEC can result in diarrheal disease accompanied by vomiting, which leads to dehydration and related complications due to excessive fluid and ion loss (Qadri et al., 2005). While the disease severity can vary, it typically only lasts between 3-4 days with a mortality rate less than 1% (Qadri et al., 2005). However, many people living in developing countries suffer greatly from ETEC infections with close to 51,000 deaths recorded

in 2016; over 18,600 of them corresponding to children under 5 years old (Khalil et al., 2018). Surviving children continually suffer from ETEC due to multiple episodes of disease per year, with high morbidity involving stunting and malnutrition, along with impaired immune system and brain development (Fischer Walker et al., 2012; Khalil et al., 2018).

1.1.3.2 Swine

In swine, an ETEC infection also often results in diarrheal disease leading to dehydration and potentially death depending on severity of symptoms. Neonates less than 4 days old commonly suffer from ETEC, with possible diarrhea showing up 2-3 hours after birth, compared to post-weaned piglets where diarrhea may show up 3 weeks, and as late as 6-8 weeks, after weaning. ETEC disease disproportionately affects neonates with a morbidity between 30-80% and a mortality of 70%, whereas for post-weaned piglets the mortality rate is 1.5-25%. In addition to dehydration, other symptoms of ETEC infection in piglets include inflamed anus, emaciation, bluish grey skin, and sunken eyes (Fairbrother & Nadeau, 2019). However, sometimes piglet death can occur from shock, without onset of any systems, likely due to lipopolysaccharide from ETEC (Gyles & Fairbrother, 2010).

1.2 Impact of ETEC on the swine industry

1.2.1 Susceptibility to ETEC

Neonatal and post-weaned piglets are vulnerable to acquiring ETEC infections, something that is not commonly seen in older pigs (Dean et al., 1989; Runnels et al., 1980). This could be due to a decrease in intestinal receptors with age; in neonates receptors are highly expressed (Fairbrother & Nadeau, 2019). Additionally, neonates have a sterile intestinal tract initially and higher stomach pH with less proteolytic activity, allowing ingested ETEC from the

environment to easily colonize without resistance (Gyles & Fairbrother, 2010). Piglets are also highly at risk for ETEC infections at weaning due to many associated stresses at this vulnerable stage. These include: leaving the sow and no longer receiving antibodies via milk, being transported into different rooms or buildings, being fed solid dry feed, and sharing a pen with different litters (Campbell et al., 2013; Fairbrother et al., 2005). Additionally, temperatures less than 25°C increase susceptibility of piglets to ETEC and worsen disease outcomes (Fairbrother & Nadeau, 2019). Furthermore, being stressed due to cold temperature and overcrowding have been seen to increase antibiotic resistance in *E. coli* isolates taken from piglets given subtherapeutic amounts of antibiotic. While the exact cause of this is unknown, it is thought these stresses may result in gut environment changes that are just more conducive to bacteria acquiring resistance (Mathew et al., 2003).

1.2.2 Economics

ETEC is not only an issue because of the suffering it causes piglets, but because of the financial burden it puts on farmers who must deal with outbreaks of ETEC in their barns. Diarrheal disease in piglets leads to economic loss due to morbidity and decreased weight, costs for treatments and preventative measures, and from mortality (Fairbrother & Nadeau, 2019). Based on the mortality rate for post-weaned piglets, this could equate to $20,000 lost annually for a herd of 500 sows (Amezcua et al., 2002). Assuming a sow has on average 22 piglets a year, the annual loss for a herd of the same size due to neonatal diarrhea was calculated to be around €68,000 (Sjölund et al., 2014).

1.3. Prevention of ETEC in swine

1.3.1 Good husbandry practices

Good hygiene should always be practiced to limit ETEC presence in barns. This involves minimizing sow fecal matter in farrowing crates through use of perforated flooring, and cleaning and disinfecting before the next sow delivers. It is additionally important that piglets are kept in a dry and warm environment, between 30-34°C for neonates and 29.5°C for post-weaned piglets to avoid stress. It is also paramount the weaning process is as stressless as possible by minimizing transportation and keeping litters together (Fairbrother & Nadeau, 2019).

Pig barn practices all center around one goal: limit the spread of disease. To help with this, barns often operates as, "all-in, all-out", facilities for biosecurity (Fairbrother & Nadeau, 2019). This means that every time a person enters and exits a barn, they shower and change into different clothes and shoes, and agree to practice frequent hand washing throughout their stay (Anderson et al., 2021; *Canadian Pork Council*, 2022). Other biosecurity measures include not permitting people who are sick on the premises and enforcing vehicle washing after visiting (*Canadian Pork Council*, 2022). It is additionally important to never allow anyone who has been in contact with other pig barns within the last 48 hours to visit. Routine maintenance to ensure cleanliness typically involves an annual cleaning and disinfecting of barns, as well as frequent cleaning of equipment (Anderson et al., 2021). This is of upmost importance when different batches of pigs are brought in to barns (Jayaraman & Nyachoti, 2017). These stringent measures are necessary for disease and pest prevention onto the farm and into barns; ultimately avoiding their spread to animals and preventing infection from taking place (Anderson et al., 2021).

1.3.2 Feed additives

The potential benefits of implementing probiotics in diet of pigs for improved health and disease protection has been explored (Dubreuil, 2017). Probiotics have been shown to prevent or alleviate diarrhea symptoms and ETEC colonization in piglets, when either *Saccharomyces cerevisiae* (Trckova et al., 2014), or several *Lactobacillus* strains (Huang et al., 2004) are administered. *Bacillus licheniformis* spores have also been observed to decrease piglet mortality from 44% to 5% by significantly reducing diarrheal symptoms (Kyriakis et al., 1999). Furthermore, providing *E. coli* stains UM-2 and UM-7 with starch together in feed has been showed to reduce the presence of ETEC in the colon and in feces (Krause et al., 2010). Though promising, more information is needed on probiotic dosing, strains, and host impacts to truly be considered for commercial use (Heo et al., 2013; Laird et al., 2021).

Supplementing organic acids or free calcium formate into feed can reduce diarrhea scores, lessens severity and length of diarrhea, and lower mortality in piglets (Bosi et al., 2007; Tsiloyiannis et al., 2001). Additionally, in some trials, 3- and 21- day old piglets can be cured of ETEC-induced diarrhea or become immune to ETEC infection entirely when given egg yolk antibodies (Marquardt et al., 1999); however this is not always the case (Chernysheva et al., 2004).

1.3.3 Vaccines

Vaccination of sows and gilts against common fimbriae virotypes of ETEC allow antibodies to be produced and past through colostrum and milk to piglets, providing a level of protection against ETEC colonization at birth (Dubreuil et al., 2016; Fairbrother & Nadeau, 2019). Because there is no cross-reactivity among fimbriae, a vaccine may need to include multiple fimbriae types to infer immunity against multiple ETEC strains (Dubreuil et al., 2016).

Passive immunity has successfully been seen to protect neonates from ETEC in a study looking at several different vaccines (H. Zhang et al., 2018). After the weaning process, piglets no longer receive maternal antibodies and are once again vulnerable to ETEC infections (Dubreuil, 2021). There are many studies that have looked at different types and preparations of vaccines to address this (Dubreuil, 2021; Melkebeek et al., 2013). Specifically, vaccination of post-weaned piglets has proven successful with vaccines Coliprotec® F4 and MucoRice-CTB against specific ETEC strains (Fairbrother et al., 2017; Takeyama et al., 2015). However, because of ETEC heterogeneity in terms of fimbriae and enterotoxins, multivalent vaccines are likely better suited to protect piglets, though it is often difficult to develop such vaccines (Dubreuil, 2021; Ruan & Zhang, 2013). With this being said, multivalent vaccines have yielded tremendous results against ETEC (Ruan & Zhang, 2013; Zhao et al., 2022).

1.4. Antibiotics

1.4.1 Usage in livestock

The largest use of antibiotics occurs in the livestock industry (Silbergeld et al., 2008) with most being administered through water or animal feed (Dall, 2020). In the past, 40-80% of feed for pigs was estimated to contain antibiotics, varying based on age (Cromwell, 2002). Antibiotics are heavily used to prevent and treat disease, improve growth, and contribute to overall animal health (Landers et al., 2012; Van Boeckel et al., 2015). Based on global usage in 2010, the use of antibiotics is anticipated to increase by 67% by 2030 to support increased food production, either through raising more animals, or with countries adopting intensive farming practices (Van Boeckel et al., 2015). The Food and Drug Administration (FDA) estimates that out of 6.1 million kilograms of antibiotics, 42% and 41% were used in swine and cattle industries respectively, whereas 3% and 10% were used in chicken and turkey industries respectively (Dall,

2020). Even though many classes of antibiotics have been used in swine, cattle, and poultry, tetracyclines are more frequently purchased (Dall, 2020; Landers et al., 2012). However, in one study, researchers testing ETEC isolates from 20 different farms showed them to be 100% resistant to tetracycline. The most commonly used antibiotic class to treat post-weaned diarrhea on these farms were aminoglycosides (Amezcua et al., 2002).

1.4.2 Growth promotion and prophylactic use

Benefits of supplementing an animal's diet with antibiotics include growth promotion, higher feed efficiency, and disease prevention (Cromwell, 2002; Hao et al., 2014). Antibiotics may reduce or remodel commensal and pathogenic bacterial populations, which could result in more nutrient adsorption for animals and lead to faster growth (Allen et al., 2013; Chattopadhyay, 2014; Wegener et al., 1999). Because of this, 10-15% less feed is required for raising animals, effectively reducing costs (Chattopadhyay, 2014). However, antibiotics have greater impacts on farms with a dirty, stressful environment and prevalent pathogenic bacteria (Cromwell, 2002), compared to a clean facility (Holt et al., 2011). Antibiotics used to protect at-risk animals against disease may be provided throughout a pig's life at subtherapeutic or therapeutic amounts (Barton, 2014). However, continual exposure to subtherapeutic levels of antibiotics is not sustainable overtime due to the strong selection pressure it presents for pathogenic bacteria to mutate and acquire resistance. Because of this, European countries have been enforcing antibiotic usage restrictions for decades, with specific countries such as Norway and Denmark completely eliminating antibiotics for growth promotion (Pokharel et al., 2020). While the FDA has banned use of antibiotics for growth promotion, prophylactic use against diseases is still allowed (Dall, 2020). Despite these efforts, when it comes to antibiotics on a

global scale, countries differ in policies, availability, and surveillance such that antibiotic usage in animal husbandry continues (Pokharel et al., 2020).

1.4.3 Resistance

Antibiotics are commonly used against ETEC infections. However, antibiotics are becoming less effective due to the prevalence of resistance against these drugs found in livestock such as pigs and cattle (Luppi, 2017; Prieto et al., 2022). In pigs, studies have shown 92.1% and 62.8% of commensal *E. coli* isolates to be resistant to at least one antibiotic or multiple antibiotics respectively (Enne et al., 2008). Furthermore, 52.5% of ETEC strains isolated from pigs without diarrheal symptoms were resistant to multiple antibiotics (Moredo et al., 2015). These antibiotics include sulfonamide, tetracycline, and streptomycin, with pathogenic *E. coli* isolates respectively, 66.82%, 68.28%, and 71.64% resistant to on average over a 13-year period (Holmer et al., 2019). Additionally, a dramatic difference in resistance among isolates was observed in spectinomycin, increasing from 0% to 59.9% from the year 2004 to 2017 (Holmer et al., 2019). Case farms in one study dealing with diarrhea in post-weaned piglets used multiple antibiotics to combat ETEC infection and consequently, more antibiotic resistance was observed on these farms (Amezcua et al., 2002). Another study found pigs fed a diet supplemented with low dose antibiotics over a 14-day period underwent a change in their intestinal microflora. This change not only led to increased bacterial resistance genes against the supplemented antibiotics, but also against another class of antibiotics not present in their diet (Looft et al., 2012). Increased resistance to other classes of antibiotics was also seen in *E. coli* and *Staphylococcus aureus* strains after being subjected to low levels of ampicillin for five days (Kohanski et al., 2010). In a recent study, 99-100% of *E. coli* isolates from healthy or sick pigs and piglets were multi-drug resistant, with colistin resistance observed in 73-95.2% of isolates from piglets under

21 weeks old (Trongjit et al., 2022). Even in cases where pigs are not exposed to antibiotics, antibiotic resistance genes are present in their fecal DNA. This shows that the swine microbiome may now inherently harbour a supply of antibiotic resistance genes due to constant antibiotic usage within the industry for over 50 years (Looft et al., 2012).

Antibiotic resistant bacteria in pigs can be transmitted from pigs to humans (Monger et al., 2021). Numerous studies have shown transmission can occur via animal contact, the environment, and through meat consumption, with resistance profiles being similar (Landers et al., 2012). Many of the same antibiotics are used in both farming and clinical settings, allowing more opportunities for resistance and cross-resistance between these drugs and bacteria to develop (Silbergeld et al., 2008; Sirichokchatchawan et al., 2021). However, meta-analysis has revealed cutting back on antibiotic usage in animal husbandry leads to less antibiotic-resistant bacteria in humans (Tang et al., 2017), giving cause to explore alternative therapeutics to deal with bacterial infections.

1.5. Bacteriophage

1.5.1 Introduction to bacteriophages

Bacteriophages (phages) are more abundant than bacteria in the biosphere with estimates of over 10^{31} compared to 10^{29} (Strange et al., 2021). Phages are viruses that specifically target and invade bacteria and can broadly be classified as either ‘virulent’ or ‘temperate’ (Principi et al., 2019). Virulent phages adopt a lytic infection cycle and immediately hijack bacterial host cell machinery upon cell entry for their propagation, causing cell lysis as progeny are released (Lin et al., 2017). This contrasts with temperate phages, which adopt a lysogenic infection cycle and rather integrate into the bacterial genome becoming a prophage, which subsequentially, leads to immunity against additional phage infections of the same type (Brives & Pourraz, 2020;

Davies et al., 2016). Temperate phages are generally excluded from phage therapy since they can increase virulence in bacterial populations via transduction and through any toxin genes they may have (Hyman, 2019). Therefore, because of their bacterial-killing ability, lytic phages are considered for therapy (Principi et al., 2019).

1.5.2 Advantages as therapeutics

Due to high bacterial specificity, phages cause minimal disruptions to natural microflora during therapy whereas antibiotics lack this specificity and may target both commensal and disease-causing bacteria, possibly leading to further infections (Khan et al., 2022; Loc-Carrillo & Abedon, 2011). Phages can also be administered at low-dosages as they further increase in numbers at infection sites, and are considered to have low inherent toxicity (Loc-Carrillo & Abedon, 2011). Research also shows potential for phages to be effective against bacteria within biofilms, clearing infections antibiotics are otherwise unable to even access (Ferriol-González & Domingo-Calap, 2020; Harper et al., 2014). Furthermore, many studies have reported synergy between phages and antibiotics when both are administered together (Diallo & Dublanchet, 2022; Łusiak-Szelachowska et al., 2022). Phages and antibiotics target bacteria through different mechanisms and receptors, allowing phages to clear antibiotic-resistant infections (Diallo & Dublanchet, 2022; Loc-Carrillo & Abedon, 2011).

1.5.3 Success as a therapeutic

1.5.3.1 In humans

While phages are not approved for clinical use in North America, approval has been given for their compassionate use when antibiotic treatments and other therapies have failed (McCallin et al., 2019). One of the most famous examples of this is when a phage cocktail was given to Tom Patterson to cure his antibiotic-resistant *Acinetobacter baumannii* infection,

completely restoring his health (LaFee & Buschman, 2017). Phages have also successfully been used to treat *Enterococcus faecalis* causing chronic prostatitis and several different diseases involving *Pseudomonas aeruginosa* including abdominal sepsis, pneumonia, intravascular coagulation, and chronic otitis (João et al., 2021; Schmalstig et al., 2021). Several studies have also shown the success of phages against skin infections including infected burns and ulcers (Principi et al., 2019; Sulakvelidze et al., 2001). Such successful outcomes are sometimes due to a combined therapy approach where phages and antibiotics are used together (Aslam et al., 2020; Kortright et al., 2019; Schmalstig et al., 2021). Unfortunately, even when phage therapy is fully approved, patients may not wish to receive it due to the many questions and uncertainties that still exist (Anomaly, 2020). Therefore, more research and case studies are needed to better define and optimize phage therapy (Schooley et al., 2017).

1.5.3.2 In livestock and other animals

Phages have successfully been used as a therapeutic to clear pathogenic *E. coli* infections in piglets, calves, and lambs showing reduced diarrheal symptoms and recovery of health (Smith & Huggins, 1983; Jamalludeen et al., 2009). Phages have also been seen to lessen symptoms from ETEC and confer a level of resistance towards infection when prophylactically administered orally or via a piglet's feed without any negative effects (Jamalludeen et al., 2009; Cha et al., 2012; Lee et al., 2017). Additionally, when administered through feed or by oral gavage, phages have been useful in preventing *Salmonella enterica* infections from establishing in pigs, chickens, and quail, due to efficient reduction of this pathogen in tissues (Ahmadi et al., 2016; Atterbury et al., 2007; Lim et al., 2012; Wall et al., 2010). Similar success has been seen with *Campylobacter jejuni* infections in chickens (Loc Carrillo et al., 2005). Research also shows the effectiveness of phage-containing eye drops for use against keratitis in horses

(Furusawa et al., 2016), as well as phage deposition into water to protect fish from *Aeromonas salmonicida* (Y. J. Silva et al., 2016). One study showed that spraying phage cocktail onto disease-ridden honey bee hives allowed the hives to make a complete recovery to health, and further protected nearby hives from becoming infected too (Brady et al., 2017). Phage therapy has also benefitted companion animals, such as dogs, for treating *Pseudomonas aeruginosa* infections (Hawkins et al., 2010; Marza et al., 2006).

1.6 Producing bacteriophage

1.6.1 Production methods

1.6.1.1 Petri Dishes

The method for producing phage on petri dishes closely resembles the double-agar overlay technique for phage enumeration. Briefly, diluted phage stock and host bacteria are mixed together in molten overlay and poured onto an underlay plate (Kropinski et al., 2009). After an overnight incubation, plates are covered with saline-magnesium (SM) buffer and kept at 4°C or 37°C with gentle shaking (Alves et al., 2014; Jamalludeen et al., 2007; Kropinski et al., 2009). After a couple hours, SM buffer and overlay are scraped into tubes for centrifugation to pellet cells and overlay, with the phage containing supernatant treated with chloroform and/or filter sterilized through a 0.2 µm filter afterwards (Alves et al., 2014; Jamalludeen et al., 2007).

Using petri dishes can be advantageous when preparing phage stocks or a small amount of phage product, but become inefficient and wasteful in scale-up when more plates and incubator space are needed. Despite this, phages have been produced on petri dishes to prepare for animal trials (Atterbury et al., 2007; Jamalludeen et al., 2009), and some phages yield higher titers on solid medium compared to in broth (Gutiérrez et al., 2011).

1.6.1.2 Shake flasks

On average, most phage production happens in shake flasks (Nabergoj et al., 2018) as it is simple and easy to carry out. Shake flasks are often used to establish a phage production strategy, with a focus of defining parameters and optimizing variables to achieve a maximum titer (Ali et al., 2019). If production is to be scaled-up to a bioreactor, it is necessary to consider conditions of the bioreactor in shake flasks to ensure scalable results (Agboluaje & Sauvageau, 2018). This was demonstrated in production of T4 phage as titers achieved in 20 mL volumes were successfully scaled up to 3 L in a stirred-tank bioreactor after conditions were first optimized in shake flasks (Ali et al., 2019). In an interview, Dr. Manual Garrido, production specialist from TechnoPhage SA stated that if a phage is "good" or "bad" in terms of its production in shake flasks, a similar outcome can be expected during scale-up using a bioreactor since the biology of phages remains the same regardless of the production method (*Cellexus: Revolutionary Biotechnology*, n.d.).

1.6.1.3 Continuous operation

Continuous operation involves two connected bioreactors, where the first continuously pumps host bacterial culture into a second already containing host bacteria and phage (Šivec & Podgornik, 2020). This second bioreactor is referred to as, 'cellstat' and operates at a lower volume, resulting in a higher dilution rate (Podgornik et al., 2015). However, the same flow rate is used through the entire system, which allows different residence times for both bacteria culturing and phage propagation (Mancuso et al., 2018). As bacterial culture is removed from the first bioreactor, fresh media is continuously added in to maintain volume and sustain exponential growth in steady state to support continued phage production in the second bioreactor (Mancuso et al., 2018; Tanir et al., 2021). Phage product continuously leaves the

second bioreactor and is collected in a vessel, which allows phage propagation of remaining phage-infected cells to be carried out and production to finish (Mancuso et al., 2018).

While producing phage continuously has the advantage of higher overall productivity, the system is expensive to set up and labour intensive due to its complexity and need for continual monitoring to make sure steady-state is maintained (Tanir et al., 2021). Furthermore, phage resistance could result if the system is disrupted and bacteria coexist with phage for a longer than desired residence time, or additionally in the holding vessel afterwards if cells remain uninfected and pass through (João et al., 2021; Tanir et al., 2021).

1.6.1.4 Two-stage, self-cycling operation

Two-stage, self-cycling operation is semi-continuous and involves cycling between two separate bioreactors: one for growing host bacteria and the other for producing phage. Cycling is initiated by changing carbon dioxide evolution rate (CER) and aided with the use of electro-optic sensors present within bioreactors (Sauvageau & Cooper, 2010). Taking the first derivative of CER (dCER) allows accurate monitoring of when bacterial cells are entering stationary growth phase and indicates conditions for cycling and harvesting in production (Sauvageau et al., 2010). To start production, bacteria are inoculated in the first bioreactor and allowed to grow until a certain dCER is reached. At this point, half of the volume is transported to the second bioreactor for infection by phage, triggering fresh medium to be pumped in to start another cycle of bacterial growth in the first bioreactor. At the second bioreactor, phage propagates within available hosts until a specific dCER is met, and then produced phage is transported to a harvest vessel. However, some phage is intentionally left in the bioreactor to allow the cycle to repeat when more host bacteria are again transferred to the second bioreactor (Sauvageau & Cooper, 2010).

The main advantage of using two-stage, self-cycling is separation of host from phage prior to infection and propagation. This allows conditions to be optimized individually to achieve best growth and production, as well as minimizes the chance of coevolution and phage resistance from developing (Agboluaje & Sauvageau, 2018; Krysiak-Baltyn et al., 2018; Sauvageau & Cooper, 2010). One disadvantage is phage production can take longer than growing host bacteria and may have to go to a harvest tank if the second bioreactor is not ready to receive more bacteria. However, this can be addressed by adding additional bioreactors for phage production to occur within (Sauvageau & Cooper, 2010).

1.6.1.5 Batch operation

In batch production, growth of bacterial host occurs in the same vessel or bioreactor as phage is produced in, and is harvested once population-wide lysis occurs (Agboluaje & Sauvageau, 2018). Batch operation is the most common method to produce phage as it is easy and cheap to carry out and yields titers in the range of 10^{10} to 10^{16} PFU/mL (João et al., 2021; Tanir et al., 2021). It is also considered a robust and relatively short process, with overall titer largely dependent on initial infection conditions, mainly multiplicity of infection (MOI), production volume, and infection load (Agboluaje & Sauvageau, 2018; Podgornik et al., 2015). Despite these advantages, batch productions often have large equipment footprints and capitol costs, require a lot of manpower, and have high downtime compared to production time, as intense sterilization and cleaning must be carried out after each run before beginning another (João et al., 2021; Sauvageau & Cooper, 2010). The cost of this escalates tremendously if different phages are consecutively produced in the same bioreactor. Alternatively, disposable bioreactors could be used, though this is suspected to provide insufficient aeration to support bacterial growth and as a result, cause a sacrifice in phage titer (Podgornik et al., 2015).

1.6.1.5.1 Cellexus CellMaker

The company Cellexus strives to overcome some of the disadvantages associated with batch operation through their bioreactor called, 'CellMaker'. The CellMaker uses single-use, plastic bioreactor bags which largely reduce downtime in between runs since no sterilization or cleaning is needed; simply autoclave and dispose of the bag. For production, bioreactor bags rest in enclosures and can accommodate volumes between 1.5-50 L. These enclosures are equipped on one side with a Peltier heater cooler, which eliminates the need for other equipment to establish a desired temperature. Additionally, aeration is provided via a sparge tube at the bottom of the bioreactor bag, which pumps in air or other gases. Sparging introduces bubbles which sufficiently mix cultures as they rise and fall over the course of production and is patented by Cellexus – 'airlift technology'. This form of aeration and mixing is a huge advantage for phage production as it replaces impellers that otherwise might cause mechanical stress and shearing to cells and phages (*Cellexus: Revolutionary Biotechnology*, n.d.).

The CellMaker has successfully been used to produce phages, in addition to being used for protein expression in *Pichia pastoris* and *E. coli*, as well as production of antibodies from murine hybridoma cells and Chinese hamster ovary cells. Companies such as Protein Pharmaceuticals, TechnoPhage, and Fixed Phage all admit to using the CellMaker for their products, but since industry is competitive, insight is not always made publicly available on how best to operate the CellMaker for maximum phage yields. However, case studies have revealed phage titers of 1 to 3 x 10^{10} PFU/mL achievable in the CellMaker, with Fixed Phage reporting a titer of 10^{13} PFU/mL (*Cellexus: Revolutionary Biotechnology*, n.d.).

1.6.2 Variables to consider

1.6.2.1 Multiplicity of infection

The multiplicity of infection (MOI) is the ratio of phage particles to bacterial host cells (Tanir et al., 2021). MOI is a critical parameter to set prior to phage production because it determines how many infection cycles take place and when to harvest (J. Silva et al., 2021). If MOI is too high, host bacteria will be lysed too quickly, and if MOI is too low, host bacteria will continually grow and reach stationary phase without detectable lysis (Rajnovic et al., 2019); both extremes could sacrifice production titer. Because of this, it is important to identify a MOI that offers a balance between growth and lysis so bacteria can continue being "phage-producing factories" (S. G. Kim et al., 2021). However, which MOI is preferential depends on infection load and can change depending if production conditions select for more virulent phage or not (Agboluaje & Sauvageau, 2018).

1.6.2.2 Bacterial inoculum

Infection load is the amount of bacterial cell culture added (% vol/vol) to start phage production and is usually between 1-5% (Agboluaje & Sauvageau, 2018; Grieco et al., 2009). Host density refers to the number of host cells present per unit volume and to start production thresholds of 7×10^3 to 3×10^4 CFU/mL have been reported (Tanir et al., 2021; Wiggins & Alexander, 1985). Higher densities and/or infection loads may be chosen to maximize phage titer since more infection cycles can occur (S. G. Kim et al., 2021; J. Silva et al., 2021), but some conditions may sacrifice titers instead (Grieco et al., 2009). Furthermore, cultures in exponential growth corresponding to 10^7 or 10^8 CFU/mL are often chosen for production (Barrow et al., 1998; Bourdin et al., 2014; Gutiérrez et al., 2011), most likely since higher titers are achieved

compared to those from cells at lag, stationary or decline phases (Bourdin et al., 2014; Sillankorva et al., 2004).

1.6.2.3 Media composition

Complex media such as Luria Bertani (LB), Blood Heart Infusion (BHI), and Tryptic Soy Broth (TSB) are commonly used to support production of a variety of phages (Ali et al., 2019; Ghorbani-Nezami et al., 2015; Kolenda et al., 2022; Nale et al., 2021). Such media often provide everything for host cells, namely nutrients and cofactors, allowing resources to be devoted to growth and replication (Z. Li et al., 2014). Depending on the study, further supplementing complex media with glucose, glycerol, or calcium may increase or decrease phage titer in production (Bourdin et al., 2014; S. G. Kim et al., 2021; Warner et al., 2014). Interestingly, having a sufficient nitrogen source in media has been found to be more important for phage infection than the carbon source (Zaburlin et al., 2017), and yet, optimizing the carbon source has been shown to improve phage titer in other productions (S. G. Kim et al., 2021). However, using complex, nutrient-rich media is expensive at large-scale and is why efforts have been made to define minimal media that support phage production (Mancuso et al., 2018; J. Silva et al., 2021). In addition, attempts should be made to avoid ingredients derived from animals to prevent possible contamination from viruses, prions, and mycoplasmas (Goodridge & Abedon, 2008).

Some phages require or benefit from supplementation of divalent cations (S. G. Kim et al., 2021; X. Li et al., 2022; Tucker, 1961), while for others they are unnecessary (Capra et al., 2006). Ion specificity does exist though, as some phages prefer one type of divalent cation over another (Chen et al., 2016; Chhibber et al., 2014) and at a specific concentration (Reddy &

McKenney, 1996). Ions have been shown to aide the phage infection cycle at adsorption and penetration of host nucleic acid (Chhibber et al., 2014).

1.6.2.4 Temperature

Temperature is often chosen to benefit the host when producing phage as it leads to larger burst sizes (Agboluaje & Sauvageau, 2018; Zaburlin et al., 2017). Because of this, *E. coli* and *Salmonella enterica* phages are typically produced at 37°C (Barrow et al., 1998; Bourdin et al., 2014; Nale et al., 2021; Wall et al., 2010). Despite this, reduced temperatures between 26-31°C have been reported to lead to high phage titer (Ali et al., 2019; Grieco et al., 2012), contradicting other studies that rather reinforce that using higher temperatures result in higher titers (Woody & Cliver, 1995). With these considerations, it has been proposed productions should utilize two different temperatures: one to benefit bacterial host growth and one for phage infection (Sillankorva et al., 2004). This is possibly because temperature impacts host physiology and can affect cell size and receptors which play a role in recognition and adsorption at the start of phage infection (Benedi et al., 1991; Sillankorva et al., 2011; Tokman et al., 2016). However, changing the temperature to 25°C can cause phages to adopt a lysogenic lifecycle compared to a highly lytic one at 37°C (Shan et al., 2014).

1.6.2.5 Harvest time

It is common practice to harvest a phage production once bacterial population-wide lysis has occurred, indicated by a noticeable reduction in turbidity or optical density (Agboluaje & Sauvageau, 2018). Harvest time depends on MOI and infection load, medium, and the phage and host system (Agboluaje & Sauvageau, 2018; Gutiérrez et al., 2018; Kolenda et al., 2022; Silva et al., 2021) and can be between 3-6 hours for a batch operation (Bourdin et al., 2014; Nale et al., 2021; Sauvageau & Cooper, 2010). Despite this, sometimes phage productions are left

overnight (Ghorbani-Nezami et al., 2015; Wall et al., 2010). However, extending production does not mean more phage will be produced, especially if peak titer is already reached, and can rather result in a titer decrease (Kolenda et al., 2022; J. Silva et al., 2021; Warner et al., 2014). Despite this, if population-wide lysis occurs early, repeated bacterial inoculations can allow for additional propagation cycles to maximize phage titer (Barrow et al., 1998).

1.6.2.6 Alternative host

Even if phages are isolated on a certain host, a different host may be better suited or preferred to produce them (Hyman, 2019). Ideally, a production host should be nonvirulent without ability to produce toxins, non lysogenic, easy to culture, and of the same species (García et al., 2019; Merabishvili et al., 2018). If a target strain does not meet these requirements, a "surrogate" host is recommended for safer large-scale production and simplified downstream processing (Gill & Hyman, 2010). Many studies have had success implementing surrogate hosts in phage production (Carlton et al., 2005; González-Menéndez et al., 2018; J. H. Kim et al., 2015; Merabishvili et al., 2012).

1.6.3 Current limitations and call to action

In theory, producing phages is straightforward: interact phages with a suitable host, allow time for propagation, and then harvest phages from lysate. Variables and parameters involved in phage production are established at laboratory scale to develop a way of maximizing phage titer; however, such methods do not necessarily scale-up to large-scale in industry (Garcia-Ochoa & Gomez, 2009; García et al., 2019; João et al., 2021; Mutti & Corsini, 2019). This is why emphasis must be put on designing a process that can be translated to a bioreactor so subsequentially, translatable phage titers are obtained (Agboluaje & Sauvageau, 2018).

Furthermore, researchers have made efforts to optimize phage production by investigating a single variable at a time (Kolenda et al., 2022; Warner et al., 2014). However, it is rather important to evaluate interactions between multiple variables to fully identify conditions that are most impactful on phage production (J. Silva et al., 2021; Warner et al., 2014). The use of computer models and equations to streamline this and predict the "best" conditions have had success even though predicted titer can be exaggerated (Grieco et al., 2012; J. Silva et al., 2021). It is also important to consider that while one variable may largely impact phage production for a specific phage-and-host system, the same variable may not for others, making it unlikely that a universal phage production strategy will be developed (J. Silva et al., 2021). Additionally, conditions that benefit bacterial host growth and optimal phage propagation within the same system can be different (Agboluaje & Sauvageau, 2018).

Even though carrying out experiments to define a scalable and robust phage production strategy can be time consuming and costly, they are necessary as demand for phage products increases (Ali et al., 2019; Grieco et al., 2012), and must be addressed by both industry and academia (García et al., 2019).

Hypothesis

Optimizing growth parameters for phage production will result in maximal phage titer and lead to an efficient and scalable production overall.

Objectives

The objectives of this work were as follows:

1. Identify growth conditions for phage production based on a high-throughput assay using 96-well plates.
2. Translate growth conditions to small and medium scale production in shake flasks and determine phage titer.
3. Upscale production to large scale, utilizing shake flasks and the CellMaker bioreactor, and compare phage titer.

CHAPTER 2.0: MATERIALS AND METHODS

2.1 Microorganisms and culture media

ETEC strain 54 (ETEC54), virotype F41:LT:STa:STb, was isolated from the small intestine of a pig at a local Manitoba farm (HyLife). ETEC54 was routinely spread plated for isolated colonies on LB agar and then further grown in various broth media by inoculation of 1 or more colonies. Cultures were shaken between 160-200 rpm (Excella® E24 Incubator Shaker, New Brunswick Scientific) unless otherwise stated for a production. Additional strains *E. coli* B (ATCC 11303) and *E. coli* C (ATCC 13706) were maintained in the same way.

A phage targeting an ETEC strain with a virotype F4:LT:STa:STb:EAST from The Reference Laboratory for *Escherichia coli* (University of Montréal) was trained to target ETEC54 by repeated passaging in a process referred to as 'phage training' (Abdelsattar et al., 2021; Borges, 2021) with a total of 12 passages being done. Following training, this phage was given the classifying number '75' at Cytophage Technologies Inc. and is referred to as 'Phage75' going forward. ETEC54 is its intended target and host. Phage75 is classified as *Myoviridae* and contains a 169 kbp dsDNA genome (information provided by Dr. Tasia Lightly).

T4 phage (ATCC 11303-B4) was used with host *E. coli* B to serve as a phage-and-host combination to compare to Phage75 and ETEC54. T4 phage is classified as *Myoviridae* and contains a 168 kbp dsDNA genome (Yap & Rossmann, 2014).

Several different media were investigated and used for bacterial growth and phage production: LB-Lennox (10 g/L tryptone, 5 g/L yeast extract, 5 g/L sodium chloride), Select-APS (2.5 g/L soy hydrolysate, 12.5 g/L yeast extract, 5 g/L sodium chloride), SM-1 novel [10 g/L casamino acids, 3.5 g K_2HPO_4, 5 g Na_2SO_4, 1 % glucose, pH = $\pm$ 0.2 (modified from Sochocka et al., 2015)], M9 minimal [(200 mL of M9 minimal salts 5x, 2 mL 1 M $MgSO_4$, 100

µl 1 M $CaCl_2$, 0.4% glucose, per 1 L (Green & Sambrook, 2012)], and Optimized M9 minimal [200 mL of M9 minimal salts 5x, 2 mL 1 M $MgSO_4$, 100 µl 1 M $CaCl_2$, 0.8% glucose, 0.4% tryptone, per 1 L (modified from Wang et al., 2018)].

When using SM-1 novel medium, bacterial cultures were started by resuspending colonies from a streak plate into fresh media with thorough mixing until a desired OD_{600} was obtained, rather than growing a culture to a certain OD_{600} (Sochocka et al., 2015).

For enumeration, all phage samples from productions were serially diluted in saline-magnesium (SM) buffer [50 mM Tris-HCl, 8.1 mM $MgSO_4$, and 100 mM NaCl, pH 7.5, (modified from Kropinski et al., 2009)].

2.2 Bacterial viability assays

Prior to phage productions, bacterial cultures in each media type were repeatedly grown to $OD_{600} \cong 0.6$. Once this desired OD_{600} was reached, cultures were put on ice and serially diluted in 0.9% saline. At least 3 dilutions from each series were spread plated on LB agar, allowed to dry, and then incubated overnight at 37°C. The next day, colonies were counted in the range of 30-300 and a CFU/mL was calculated for use in MOI calculations in phage productions.

Additionally, bacterial cultures used to start phage productions on larger scale were enumerated the same way to make sure an intended MOI was indeed achieved.

2.3 Preparation of phage for electron microscopy (EM) imaging

Phage stock was diluted to approximately 10^8 PFU/mL in 3 mL of SM buffer. Based on Bourdin et al., 2014, phages were purified using a sucrose cushion. To achieve this, 14 mL of chilled 20% sucrose solution was added to a centrifuge tube with 3 mL of diluted phage sample gently layered on top. Fresh SM buffer was additionally added to fill the tube (about 3 mm from

the edge) to prevent potential collapse of tube during spinning. Samples were spun at 27,000 rpm (Beckman-Coulter Optima XE ultracentrifuge, rotor SW32Ti) for 4 hours at 5°C, with centrifuge tubes decanted afterwards and inverted to dry (Bourdin et al., 2014). The phage pellet was reconstituted in 500 µL of SM buffer at 4°C and further purified for EM imaging based on the protocol by Ackermann, 2009. Briefly, phages were spun down at 21,000 x g for 60 minutes at 4°C. The supernatant was then removed and replaced with 0.1 M ammonium acetate, pH 7, and then centrifuged again at 21, 000 x g for 60 minutes at 4°C twice (Ackermann, 2009). The resulting phage pellet was resuspended in 15 µL nuclease-free water and then sent away for electron-microscopy imaging (The Canadian Centre for Electron Microscopy, McMaster University). Phage morphology was analyzed using ImageJ software (Schneider et al., 2012).

2.4 Plate assay for high-throughput screening

To screen production variables, a 96-well plate assay was developed based on Rajnovic et al., 2019. Bacterial cultures were grown to an $OD_{600} \cong 0.6$ and then diluted 1/10 to achieve $\cong 10^7$ CFU/mL. Phage stock was then diluted, using the same medium as bacteria were grown in, to appropriate MOI(s). As stated, 20 µL of diluted bacterial culture and 20 µL phage dilution were added to 160 µL of media for each experimental condition screened (Rajnovic et al., 2019). The total volume in each well was 200 µL, and all plates contained media and bacterial controls. Sterile water was added to wells along the plate perimeter for hydration throughout the assay. Plates were incubated overnight in a plate reader (Biotek EPOCH 2 or Biotek Synergy HTX) set to continuous orbital shaking at 205 cpm (5 mm) with OD_{600} measurements taken every 15 minutes.

Several different MOIs were investigated for media LB, APS, SM-1, M9 minimal, and Opt. M9, in the range of 1 to 10^{-7}. The MOI conditions predicted to be the "best" for phage

production were further screened at temperatures 28°C, 33°C, 37°C and 40°C. Furthermore, concentrations of $CaCl_2$ and $MgSO_4$ in the range of 0 mM – 10 mM were screened in LB medium using a 10^{-4} MOI. 1 M solutions of $CaCl_2$ and $MgSO_4$ were diluted in LB to achieve desired concentrations before being added to plates for screening.

2.5 Titration conditions for Phage75 and T4 phage

The double-agar overlay technique was used to enumerate phage and was adapted from Kropinski et al., 2009. Phage samples were serially diluted in SM buffer and depending on the phage sample and intended host, an appropriate bacterial colony (ETEC54 or *E. coli* B) was inoculated into LB broth and grown to an $OD_{600} \cong 0.8$. At OD_{600}, the culture was put on ice. LB, 0.2% agarose (for Phage75) or LB, 0.6% agar (for T4 phage) overlay was microwaved on high until boiling with intermittent shaking until bubbling was observed. Then, overlay was aliquoted in 3 mL volumes into 5 mL culture tubes and maintained within a temperature range of 46°C-50°C using a heat block. 200 µL of respective host bacteria and 100 µL of phage sample dilution were added to 3 mL overlay and poured over petri dishes containing solidified LB 1.5% agar underlay. At least 3 dilutions from each dilution series were plated (Kropinski et al., 2009).

Alternatively, 200 µL of host bacteria were added to 3 mL of melted overlay and poured over petri dishes containing solidified LB 1.5% agar underlay and allowed to solidify for about 15 minutes. Following this, 10 µL drops of diluted phage samples were spotted at least in triplicate for each dilution plated onto overlay and allowed to dry. This form of plating was primarily used to enumerate the timepoint samples gathered during the harvest time experiments and for back-tittering phage at the 100 mL and 1 L scales to ensure the intended MOI was indeed achieved to start a phage production (Sections 3.7 to 3.9, and 3.12).

Once plates were dry, they were inverted and incubated overnight at 37°C. The next day, plaques were typically counted in the range of 0-50 for spots, and between 30-500 for full plates (Kropinski et al., 2009). However, due to the small plaque size of Phage75, plaque numbers up to 800 were easily counted if necessary. All titers were calculated as PFU/mL and then transformed to $\log_{10}$(PFU/mL) for graphing and analysis.

2.5.1 Efficiency of plating (EOP)

Phage75 stock was serially diluted and titrated on ETEC54, *E. coli* B, and *E. coli* C as described in Section 2.5. EOP was calculated by dividing the titer obtained on *E. coli* C and *E. coli* B by the titer obtained on ETEC54 and then multiplied by 100.

2.6 Plate staining

Titration plates were stained following a protocol adapted from Baer & Kehn-Hall, 2014 to enhance plaque visualization. Briefly, crystal violet was dissolved in 20% ethanol at a 1% concentration. This crystal violet solution was then furthered diluted in MilliQ water to achieve a concentration of 0.01%. To stain a titration plate, 3 mL of 0.01% crystal violet solution was deposited and swirled regularly until plate was stained a deep purple colour, taking approximately 45-90 seconds. The dye was then removed and plates were rinsed several times with MilliQ water before being flooded again with water and left for about 5 minutes with occasional swirling. Water was then removed and plates were inverted and imaged (Baer & Kehn-Hall, 2014).

2.7 Determination of initial infection conditions for production

Initial infection conditions, infection load (IL) and MOI, were explored in small-scale using shake flasks, where IL is the amount of host cell culture present on a % vol/vol basis

(Agboluaje & Sauvageau, 2018). ILs of 1-5%, 7%, 10%, and 20% were investigated for three different MOI conditions: 10^{-3}, 10^{-4}, and 10^{-5}. Briefly, 10 mL of LB broth was added to eight, 50 mL shake flasks labelled for each IL to be tested. From each flask, the IL volume was then removed, and flasks were left to pre-heat at 37°C until production. A culture of ETEC54 was started by inoculating a colony into approximately 6 mL of LB broth and left to grow in a shaking incubator until an $OD_{600} \cong 0.6$, corresponding to approximately 10^8 CFU/mL. Once the ETEC54 culture reached the target OD_{600}, IL volumes were added to each of the shake flasks along with 100 µL of diluted Phage75 in order to appropriate desired MOI conditions. Shake flasks were then incubated for 8 hours, shaking at 200 rpm (VWR® Incubating Mini Shaker) at 37°C. Following production, 1 mL samples were harvested from each shake flask and filter sterilized using 0.2 µm syringe filters (Agboluaje & Sauvageau, 2018). Samples were enumerated as described in Section 2.5.

2.8 Productions in shake flasks

All productions were based on Agboluaje & Sauvageau, 2018 and modified according to the variable and volume scale being tested. However, for all productions a 4% IL was used. To start a production, 1-2 bacterial host colonies were inoculated in fresh media and allowed to grow at 37°C in a shaking incubator (Excella® E24 Incubator Shaker) until an $OD_{600} \cong 0.6$ was reached, which corresponded to about 10^8 CFU/mL for ETEC54; 10^7 CFU/mL for *E. coli* B. When working with *E. coli* C, the OD_{600} desired was approximately 0.6 in APS and 0.8 in LB and corresponded to around 5.5 x10^7 CFU/mL in each medium.

While cultures grew, phage stock was diluted in microcentrifuge tubes using the same production media to appropriate a desired MOI. Once bacteria reached desired OD_{600}, bacteria and phage were added to shake flasks containing the same pre-warmed media that had been used

to grow bacteria and dilute phage (Agboluaje & Sauvageau, 2018). Productions were carried out at 37°C unless otherwise stated.

At 10 mL production scale, the volume of bacteria added to start production was considered in the total volume, but the volume of phage was not. Productions were run using a VWR® Incubating Mini Shaker set at 200 rpm and harvested after 8 hours. To harvest, a 1 mL sample from each flask was filter sterilized using a 0.2 µm syringe filter and kept at 4°C for future enumeration and titer determination.

At 100 mL and 1 L scale, both bacteria and phage additions to start a production were considered in the total volume. These volumes were 4 mL bacterial culture to 1 mL phage dilution and 40 mL bacterial culture to 10 mL phage dilution for 100 mL and 1 L scales respectively. Productions at 100 mL were shaken at 160 rpm whereas those at 1 L were shaken at 120 rpm (Excella® E24 Incubator Shaker). Shaking speeds were adjusted lower at these scales to avoid aggressive foaming and bubbling that resulted in these larger volumes.

2.8.1 Harvest time

Once a production was set up with bacteria and phage additions, the flask was swirled several times and a 3 mL sample was removed. From this 3 mL sample, a 1 mL sample was filter sterilized using a 0.2 µm syringe filter for phage titration, with the rest returned to the flask. This was ‘time zero’. The flask was then incubated at 37°C with appropriate shaking depending on volume (100 mL or 1 L) and removed every hour to filter sterilize another 1 mL sample for titer determination. This was continued for 8 hours.

At the 1 L scale, OD_{600} measurements were additionally taken every 30 minutes (Biochrom WPA CO8000 cell density meter). If samples were very turbid, a 1/10 dilution was

made prior to taking this OD_{600} measurement. After 8 hours, the production flask was left shaking overnight with an additional timepoint sample being taken at 22 hours.

For productions using *E. coli* C, timepoint samples were only collected for up to 5 hours.

2.8.2 Susceptibility of residual bacteria

Samples of ETEC54 were removed at 22 hours following a harvest time experiment and streaked out for isolated colonies. Three colonies from each run were inoculated into fresh LB medium and grown until an $OD_{600} \cong 0.8$. Titration conditions were as described in Section 2.5, where Phage75 stock and Phage75 production samples were serially diluted in SM buffer and spotted on bacterial lawns of residual ETEC54 from production. ETEC54 from these colonies were classified as being susceptible to phage if after an overnight incubation at 37°C any plaques were observed across dilutions 10^{-6} and 10^{-7}. These dilutions were chosen because Phage75 titer was otherwise countable at these dilutions.

2.9 CellMaker bioreactor for Phage75 production

The CellMaker 8 L enclosure (Cellexus, Scotland) was preheated to 37°C. One end of autoclaved tubing was attached to the media input tube on a bioreactor bag and the other, to a Vesapor pleated 0.2 – 0.8 µm filter. The bag was then carefully fitted into the CellMaker enclosure and 3 L of LB medium were pumped into it using a peristaltic pump (MasterFlex, Germany). After media was pumped into bag, the media input tube was pinched, capped, and tied up against the sparge tube to avoid any potential leakage (Appendix A.4 A). The airflow for the sparge tube was set to 0.25 Lpm.

ETEC54 culture was grown and Phage75 dilutions were done as described in Section 2.8; however, a loopful of streaked out ETEC54 was used to start the culture and calculations were

adjusted to accommodate an increased production volume. Once ETEC54 was at an $OD_{600} \cong$ 0.6, an aliquot was removed and kept on ice to carry out viability assays (Section 2.2). 120 mL of ETEC54 culture and 30 mL of Phage75 dilution were then added to the CellMaker by syringes to appropriate a MOI of 10^{-4}. Samples were removed in triplicate from a sampling port on the bioreactor bag at 3-hour and 4-hour timepoints for titration and enumeration (Section 2.5).

2.10 Statistical analysis

One-way ANOVA was routinely performed using an alpha threshold of 0.05 with a 95% confidence interval to analyze obtained phage titers from production. This test was always followed up with a Tukey multiple comparisons test, where mean titers were directly compared to see if existing differences were significant under the set parameters. These data analysis were carried out using GraphPad Prism (Dotmatics, San Diego).

CHAPTER 3.0: RESULTS

3.1 Morphology comparison of Phage75 and T4 phage

Based on morphology, Phage75 and T4 phage appear to resemble one another (Appendix A.1). The phage head lengths and widths were measured to be 105.6 ± 11.0 nm and 80.5 ± 6.0 nm for Phage75, and 105.2 ± 5.0 nm and 78.1 ± 8.8 nm for T4 phage. The length of the tail sheath was 121.0 ± 22.8 nm and 89.9 ± 11.0 nm for Phage75 and T4 phage respectively.

3.2 Screening MOI and possible harvest time

Bacterial growth curves were carried out for ETEC54 in the presence of Phage75 at different MOI in several media (Figure 1). To identify which MOI were beneficial for phage production, the balance between ETEC54 growth (indicated by increasing OD_{600}) and its lysis (indicated by decreasing OD_{600}) was analyzed. Overall, screening assays done in LB (Figure 1 A) and APS (Figure 1 B) media appeared similar and predicted a MOI of 10^{-4} and possibly 10^{-5} to be useful for production. On the contrary, MOI conditions 10^{-2} and 10^{-3} seemed to cause bacterial lysis too quickly, whereas hardly any lysis was indicated for MOI conditions 10^{-6} and 10^{-7}. These trendlines closely resembled the bacterial control, suggesting unimpeded ETEC54 growth despite addition of Phage75. When considering a 10^{-4} MOI however, harvest time could also be approximated by identifying when OD_{600} most resembled the media blank, suggesting population-wide lysis had occurred. Harvest times of 5.5 hours and 4.5 hours were predicted for productions carried out in LB and APS respectively.

Opt. M9 medium was chosen for investigation since it has been shown to support bacterial growth similar to LB media (Wang et al., 2018). In Opt. M9 (Figure 1 C), 10^{-3} MOI was predicted to work best for Phage75 production, however due to the growth trendline, harvest

time could not be predicted as OD_{600} did not reach media blank levels throughout the experiment.

SM-1 was explored because titers of 10^{16} PFU/mL have been reported using this medium (Sochocka et al., 2015). A 10^{-3} MOI and 8-hour harvest time were predicted for productions in SM-1 (Figure 1 D). However, ETEC54 did not reach the same peak OD_{600} in this medium as in other media (Figure 1 A-C) before succumbing to Phage75 lysis, suggesting fewer available hosts and potentially less Phage75 produced.

In M9 minimal, only a MOI of 1 was observed to show reduction in ETEC54 in the presence of Phage75, with other tested MOIs resembling the bacterial control (Figure 1 E). To see if this outcome was Phage75 specific, the assay was repeated using the phage and host combination, T4 phage and *E. coli* B (Figure 1 F). The results show *E. coli* B was sufficiently reduced by T4 phage at much lower MOI, like 10^{-3}, suggesting M9 minimal is better able to support T4 propagation and production and likely not those of Phage75.

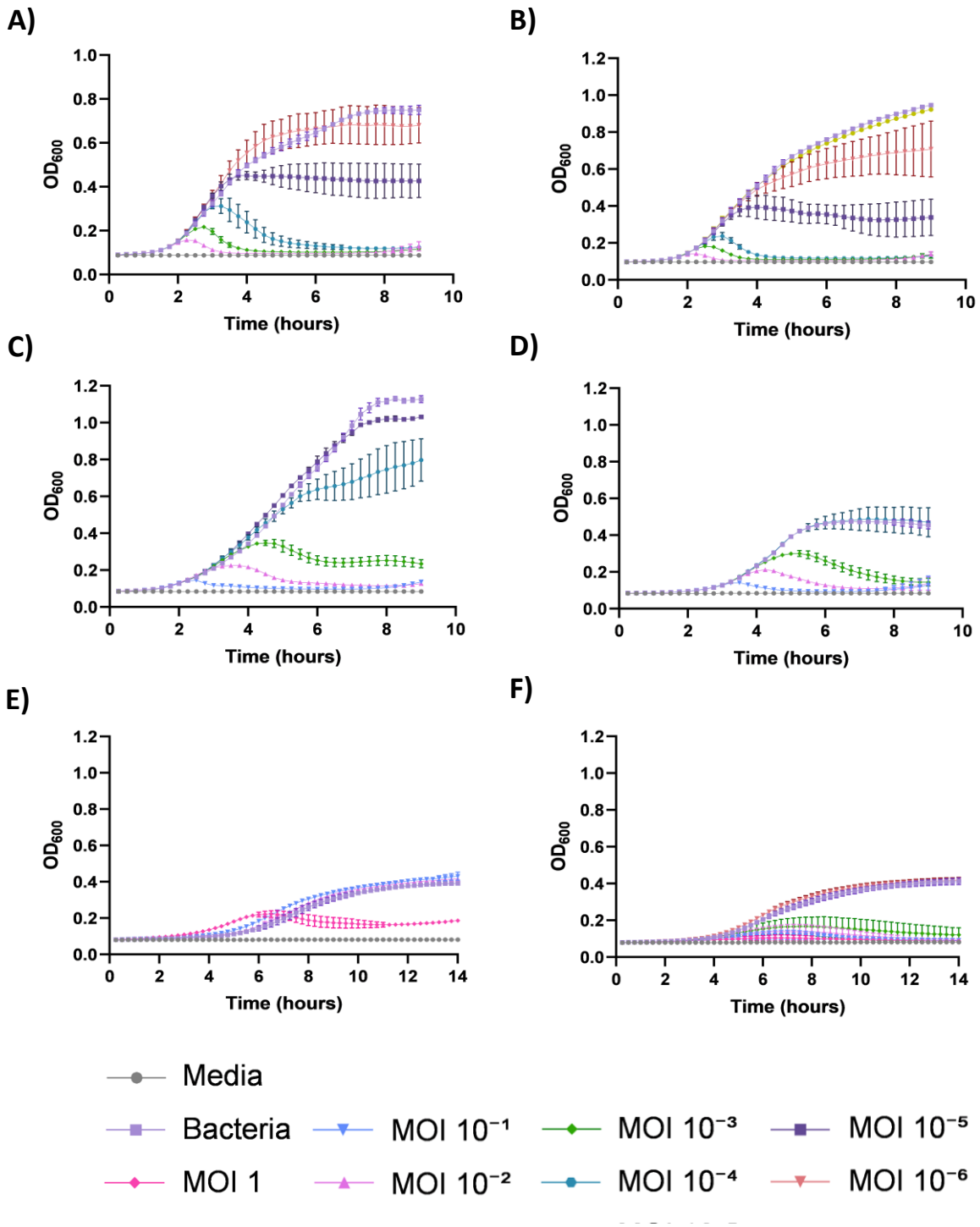


Figure 1. Effect of media on bacterial growth in the presence of phage at different MOI. **A-E)** ETEC54 and Phage75; **F)** *E. coli B* and T4 phage**.** Media types: **A)** LB; **B)** APS; **C)** Opt. M9; **D)** SM-1; **E/F)** M9 minimal. OD_{600} measurements were taken every 15 minutes in a plate reader incubated at 37°C with orbital shaking of 205 cpm (5 mm). Error bars show standard deviation from the mean (n = 3).

3.3 Infection load and MOI investigation in small-scale production

Infection load (IL) is defined as the volume of host cell culture present (% vol/vol) and together with MOI, is considered an initial infection parameter to establish and set before scaling up phage production. Since IL reflects the number of host cells present at the start of a production, this influences the dynamics between cells and phages and may affect how many cycles of phage replication occur prior to harvest (Agboluaje & Sauvageau, 2018). However, regardless of infection load, a similar Phage75 titer was achieved for each MOI condition tested (Figure 2). MOIs 10^{-3} and 10^{-4} resulted in titers in the mid 9 $\log_{10}$(PFU/mL) whereas for MOI 10^{-5}, titers in the high 9 $\log_{10}$(PFU/mL) and low 10 $\log_{10}$(PFU/mL) were achieved. However, large batch to batch variability in the 10^{-5} condition in the range of 0.15-0.34 $\log_{10}$(PFU/mL) for IL 1-5%, selected against its use for future productions, despite a higher titer. Additionally, since 10x more phage was needed to establish a 10^{-3} MOI compared to a 10^{-4} MOI, a 10^{-4} MOI was chosen to move forward with as the two conditions yielded similar overall titers. While IL 1-5% and 7% had similar titers for a 10^{-4} MOI, a 4% IL was chosen. A 4% IL resulted in the highest titer for MOI 10^{-4} with small batch to batch variability. In addition, several IL percentages with different MOI were tested for T4 phage and showed out of those tested, a 4% IL with 10^{-4} MOI were good choices for production of this phage as well (Appendix A.2).

3.4 Screening ion supplementation in LB

Supplementation of $CaCl_2$ had minimal impact on ETEC54 bacterial growth, as similar trends were observed with and without it (Figure 3 A). This contrasted with supplementation of $MgSO_4$ which seemed to support continual bacterial growth exceeding the duration of the experiment; without $MgSO_4$ growth settled in a plateau (Figure 3 B).

With the addition of Phage75, ETEC54 bacterial reduction curves were similar, with no obvious ion or ion concentration predicted to yield the most phage (Figure 3 C and D). However, comparison of highest achieved OD_{600} to the lowest achieved OD_{600} for each condition allowed insight into the balance between bacterial growth and lysis; a larger difference suggestive of more phage produced. One-way ANOVA showed no significance ($p = 0.9951$) between OD_{600} differences for $CaCl_2$ supplementation assays. However, one-way ANOVA followed by a Tukey multiple comparisons test showed there was some significance between 0 mM and 6 mM ($p = 0.0166$) and between 0 mM and 8 mM ($p = 0.0355$) $MgSO_4$ supplementation. Even with potential gain in titer, addition of $MgSO_4$ was predicted to extend harvest time which could make production less efficient.

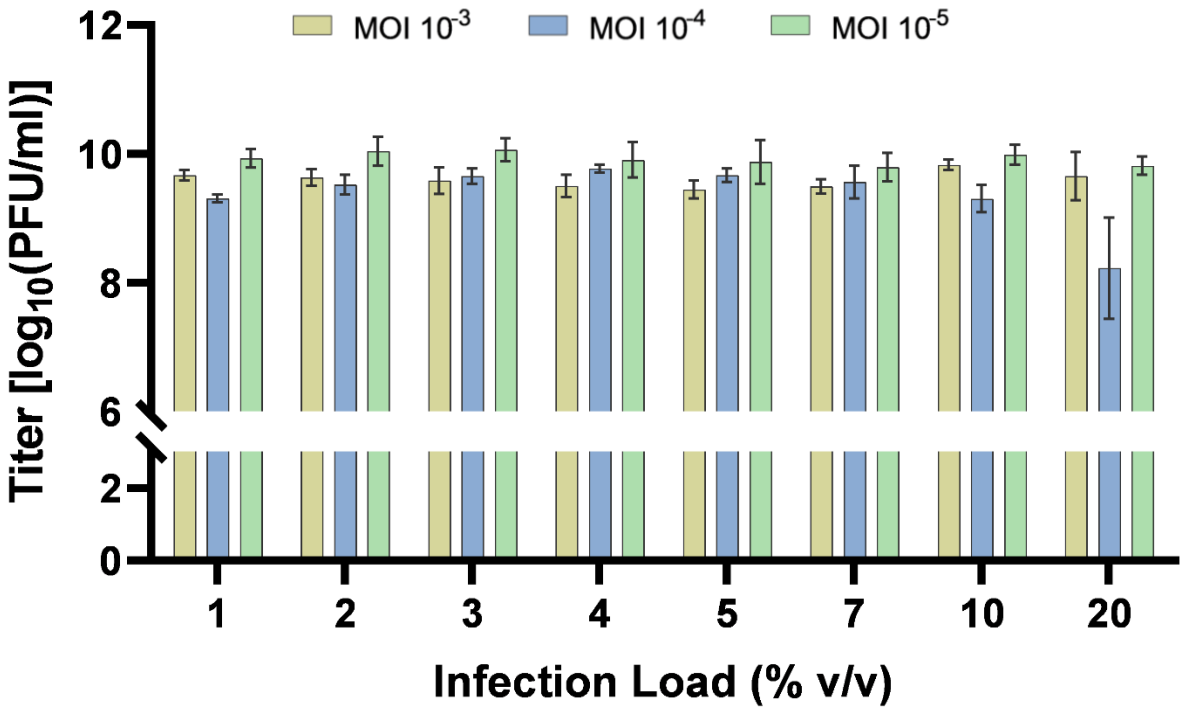


Figure 2. Titer comparison between different MOI conditions and infection loads for Phage75 production in ETEC54 host. Productions were carried out in 10 mL of LB incubated at 37°C with shaking at 200 rpm (VWR® Incubating Mini Shaker) and harvested after 8 hours. Error bars show standard deviation from the mean (n = 3-4).

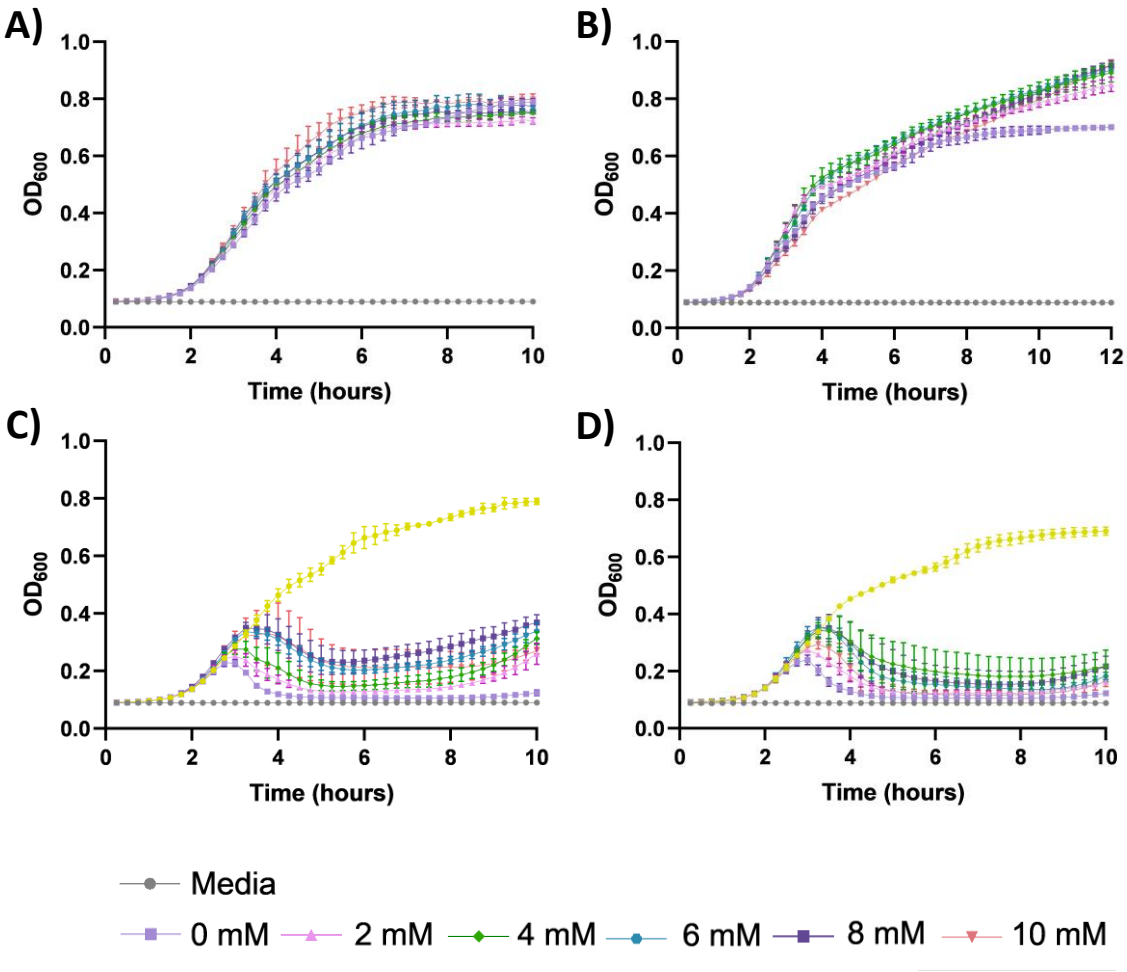


Figure 3. Growth curves of ETEC54 in LB supplemented with ions. A) ETEC54 with $CaCl_2$; **B)** ETEC54 with $MgSO_4$; **C)** ETEC54 and Phage75 with $CaCl_2$; **D)** ETEC54 and Phage75 with $MgSO_4$. For graphs C) and D), a 10^{-4} MOI was used with bacterial control (no added phage or ion) being shown in yellow. OD_{600} measurements were taken every 15 minutes in a plate reader incubated at 37°C with orbital shaking of 205 cpm (5 mm). Error bars show standard deviation from the mean (n = 3).

3.5 Screening temperature

Additional screening assays were carried out in 96-well plates using predicted MOI for each media type (Section 3.2; Figure 1) at temperatures: 28°C, 33°C, 37°C, and 40°C (Figure 4). In LB and APS (Figures 4 A and B respectively) trendlines were similar for bacterial controls as well as phage conditions at 37°C and 40°C, with harvest time predicted around 4.5-5 hours. However, at 33°C ETEC54 was observed to reach higher OD_{600} before lysis, which could potentially mean more available host to support more phage production. Despite this, 33°C extended predicted harvest time to 6.5-7 hours in LB and affected population-wide bacterial lysis in APS. At 28°C, ETEC54 was less affected by Phage75 in LB than in APS indicated by less bacterial reduction.

These results contrast assays carried out in Opt. M9 (Figure 4 C), where 33°C offered a better balance between ETEC54 growth and lysis than 40°C did and seemed comparable to the reduction curve observed at 37°C. Additionally, 33°C showed a 6-hour harvest time for productions in Opt. M9. Interestingly, for temperatures 33°C, 37°C, and 40°C ETEC54 bacterial controls reached a plateau in OD_{600}; something not seen in LB or APS media over the timeframe of the experiment.

No improvement was observed for assays using SM-1 media when temperature was changed from 37°C (Figure 4 D). Since small-scale productions in SM-1 at 37°C were on average 1 log less than otherwise achieved in other media at small-scale (Figure 5 A), SM-1 was removed from study as a media option for Phage75 production.

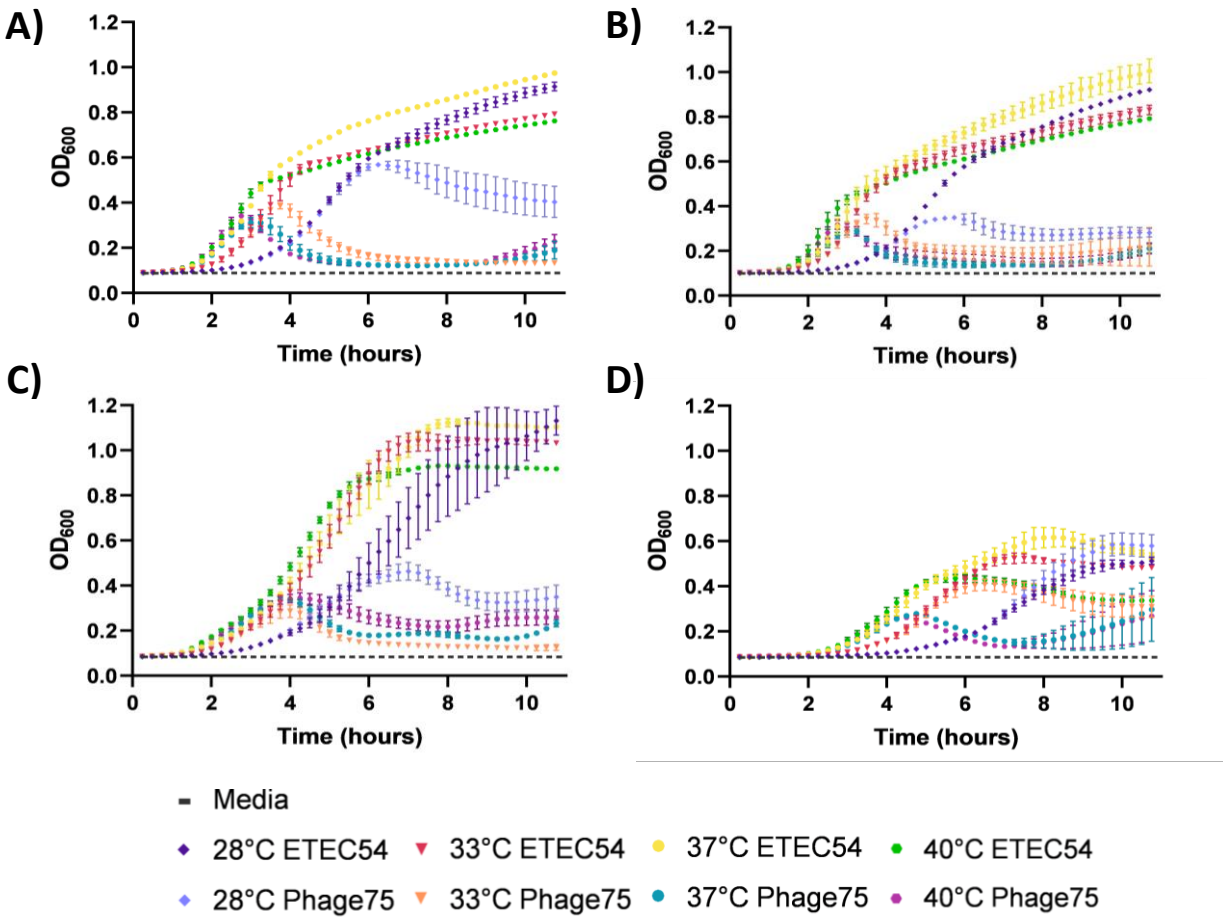


Figure 4. Growth curves of ETEC54 in the presence of Phage75, in different media, at different temperatures. Media (MOI used): **A)** LB (10^{-4}); **B)** APS (10^{-4}); **C)** Opt. M9 (10^{-3}); **D)** SM-1 (10^{-3}). OD_{600} measurements were taken every 15 minutes in a plate reader incubated at 37°C with orbital shaking of 205 cpm (5 mm). Error bars show standard deviation from the mean (n = 3).

3.6 Small-scale production

Using MOI predicted from screening assays mentioned in Section 3.2, 10 mL productions were carried out for each medium and harvested at 8 hours (Figure 5 A). A titer of 10.10 ± 0.12 $\log_{10}$(PFU/mL) was achieved in Opt. M9 medium and a titer in the range of $9.18\text{-}9.5 \pm 0.07\text{-}0.18$ $\log_{10}$(PFU/mL) was achieved in LB, APS, and M9 minimal. A titer of 8.38 ± 0.04 $\log_{10}$(PFU/mL) was achieved in SM-1. One-way ANOVA followed by Tukey multiple comparisons test revealed no significance ($p > 0.05$) between titers achieved in LB to either of the APS conditions or M9 minimal, nor among APS conditions themselves. However, the difference in titer between LB and Optimized M9 was significant ($p = 0.0002$). Additionally, significance was observed between titer achieved in LB to that in SM-1, as well as between M9 minimal and Opt. M9 ($p < 0.0001$). OD_{600} measurements were additionally recorded following productions and were 0.10 ± 0.03 (LB), 0.14 ± 0.02 (APS, MOI 10^{-4}), 0.18 ± 0.04 (APS, MOI 10^{-5}), 2.17 ± 0.39 (SM-1), 0.03 ± 0.01 (M9 minimal), 0.24 ± 0.02 (Opt. M9).

Another series of small-scale productions were carried out to investigate temperature and supplementation of ions (Figure 5 B). From these results, adding 6 mM $MgSO_4$ to LB resulted in a titer of 10.20 ± 0.01 $\log_{10}$(PFU/mL), where there was a significant difference between its titer and that determined in LB without $MgSO_4$ ($p = 0.0029$) as well as between it and APS with and without 6 mM $MgSO_4$ ($p = 0.0029$ and $p = 0.0070$ respectively). Despite prediction that 33°C may yield more phage in productions carried out in either LB or APS (Section 3.5, Figure 4), there was no significance observed between the different titers ($p > 0.9999$ and $p = 0.7625$ respectively). Furthermore, using 33°C for production in Opt. M9 resulted in a reduced titer of 9.91 ± 0.05 $\log_{10}$(PFU/mL) compared to what was achieved at 37°C (Figure 5 A).

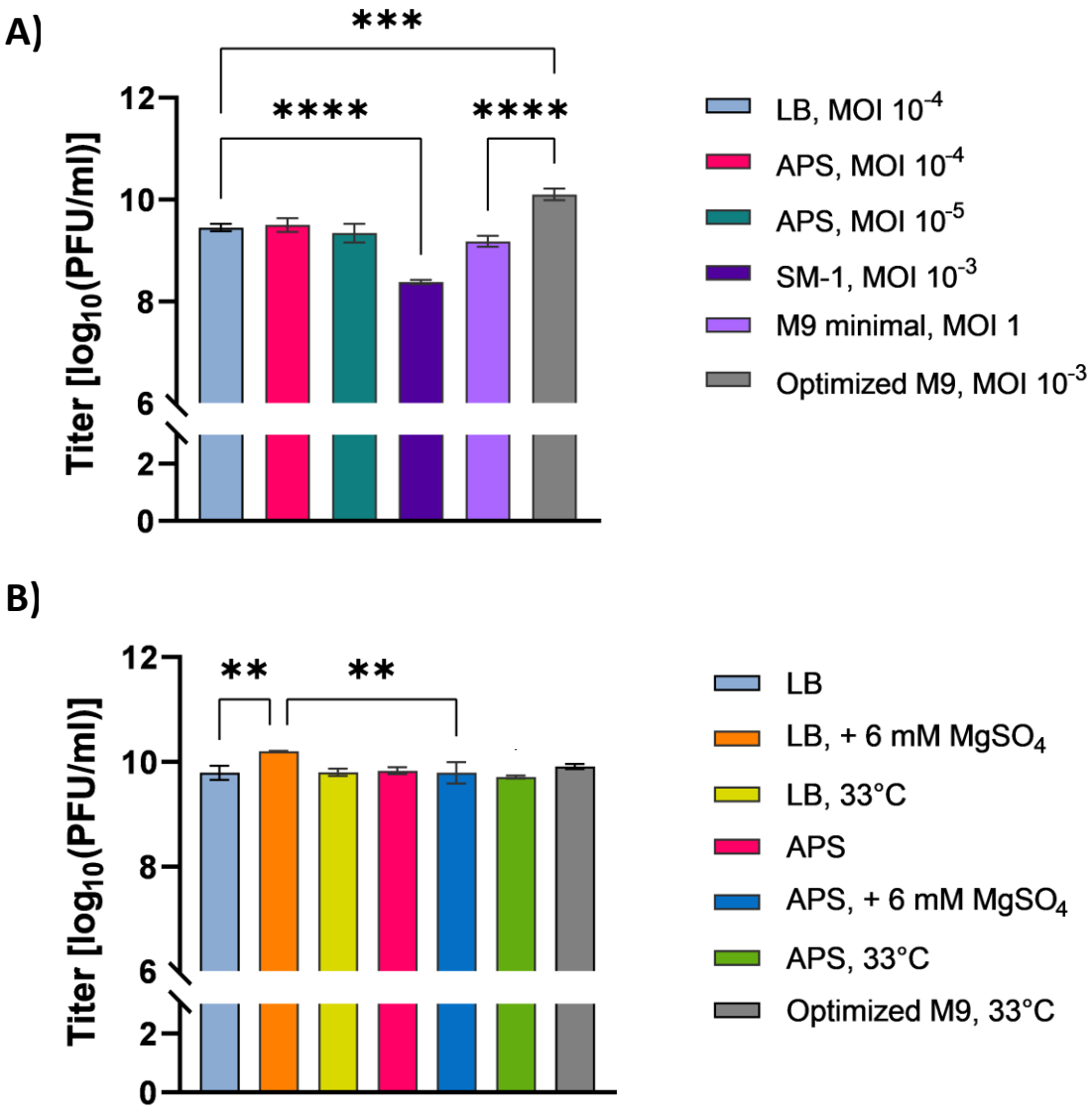


Figure 5. Titers for small-scale productions of Phage75 with ETEC54. Overall production conditions: 4% infection load, 10 mL media, 200 rpm (VWR® Incubating Mini Shaker), 8-hour harvest time, 37°C incubation (unless otherwise stated). **A)** Comparison of "best" MOI condition(s) for each medium type. **B)** Comparison of 6 mM $MgSO_4$ ion supplementation and temperature decrease to 33°C for most promising medium types: LB; APS; Opt. M9. Media (MOI): LB (10^{-4}); APS (10^{-4}); Opt. M9 (10^{-3}). Error bars show standard deviation from the mean (n = 3). Statistics performed using one-way ANOVA with Tukey multiple comparisons test, **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$.

3.7 Investigation into harvest time

While screening assays suggested potential harvest times, bacterial reduction curves remained in plateau for several hours after assumed lysis (Figure 1, 3, and 4) raising the question of when a good time to end production is. Over an 8-hour production in 100 mL of LB, timepoint samples taken at 6 hours revealed peak titers of 9.85 ± 0.29 $\log_{10}$(PFU/mL) and 9.99 ± 0.01 $\log_{10}$(PFU/mL) were achieved for Phage75 at MOIs 10^{-5} and 10^{-4} respectively (Figure 6). However, one-way ANOVA and Tukey multiple comparisons test revealed no significance ($p > 0.97$) in titer among samples taken over 4-8 hours, showing extended production was unnecessary. While a higher titer of 10.56 ± 0.11 $\log_{10}$(PFU/mL) was achieved for T4 phage at 5 hrs into production, one-way ANOVA and Tukey multiple comparisons test showed no significant difference ($p > 0.9999$) among titers for samples taken within 4-8 hours.

At 1 L scale, production of Phage75 was seen to reach a peak titer of 9.83 ± 0.06 $\log_{10}$(PFU/mL) at 4 hours, coinciding with additional OD_{600} measurements which showed population-wide lysis of ETEC54 had occurred (Figure 7 A). Furthermore, one-way ANOVA and Tukey multiple comparisons test revealed no significant difference among titers from samples gathered 3-8 hours into the production ($p = 0.3068$ to > 0.9999). The same trend was observed for T4 phage, however peak titer of 10.56 ± 0.03 $\log_{10}$(PFU/mL) was rather reached at 6 hours (Figure 7 B) with no significant difference among titers between 4-8 hours of the production ($p = 0.9975$ to > 0.9999). Additionally, extending production overnight to 22 hours did not result in higher phage titer, 9.29 ± 0.22 and 10.41 ± 0.02 for Phage75 and T4 phage respectively, but rather a marked increase in OD_{600}, suggestive of bacterial growth. Images taken every hour at sampling reflected results and showed production media clear at start, becoming increasingly turbid at 1 hour and 2 hours, then less turbid at 3 hours, with clearing at 4

hours (Figure 8). Production media was observed to remain clear up until 8 hours, whereas at 22 hours, it was turbid and opaque. Bacterial samples removed from production at this time were tested for susceptibility to Phage75 stock as well as Phage75 produced from the same run. As summarized in Appendix A.3, residual bacteria represented a “mixed bag”, where some colonies were susceptible to Phage75 and others were not, indicated by the presence of plaques across two different dilutions.

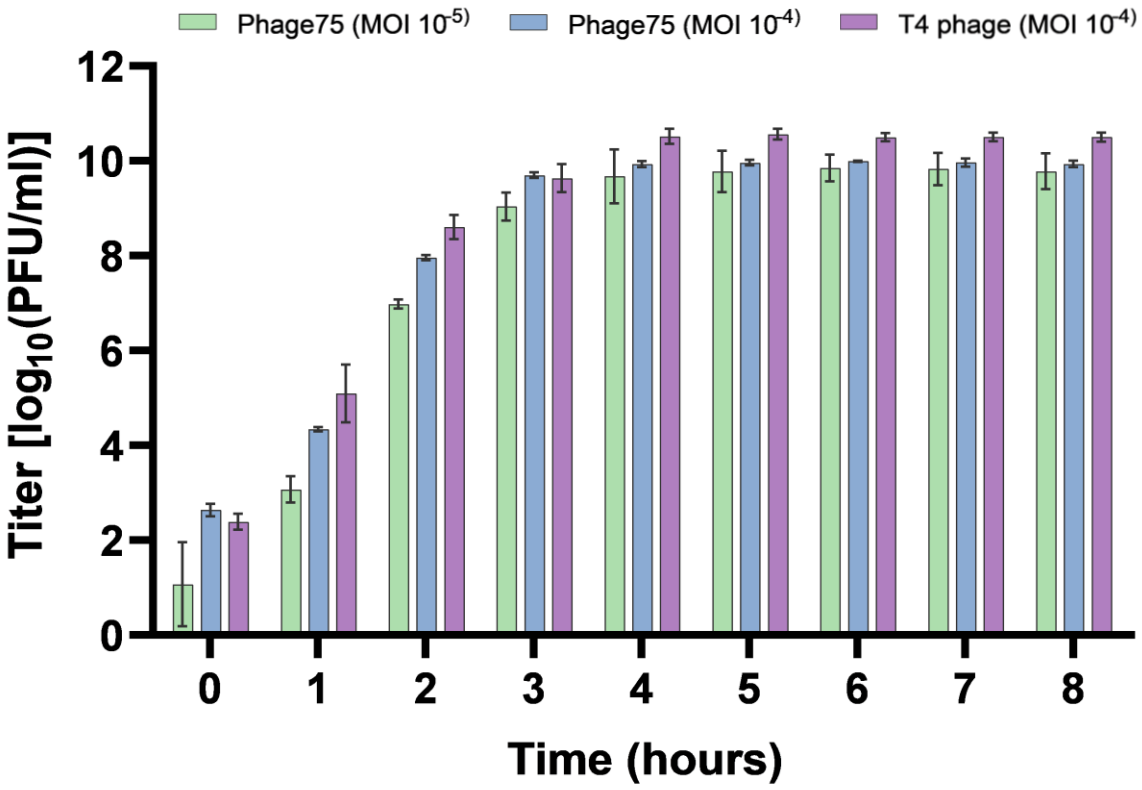


Figure 6. Comparison of phage titer throughout production at 100 mL scale in LB medium. All productions had a 4% infection load and a 37°C incubation. A 1 mL sample was removed from shake flasks every hour and filter sterilized for titer determination. Error bars show standard deviation from the mean: Phage75, MOI 10^{-5} (n = 5); Phage75 and T4 phage, MOI 10^{-4} (n = 3).

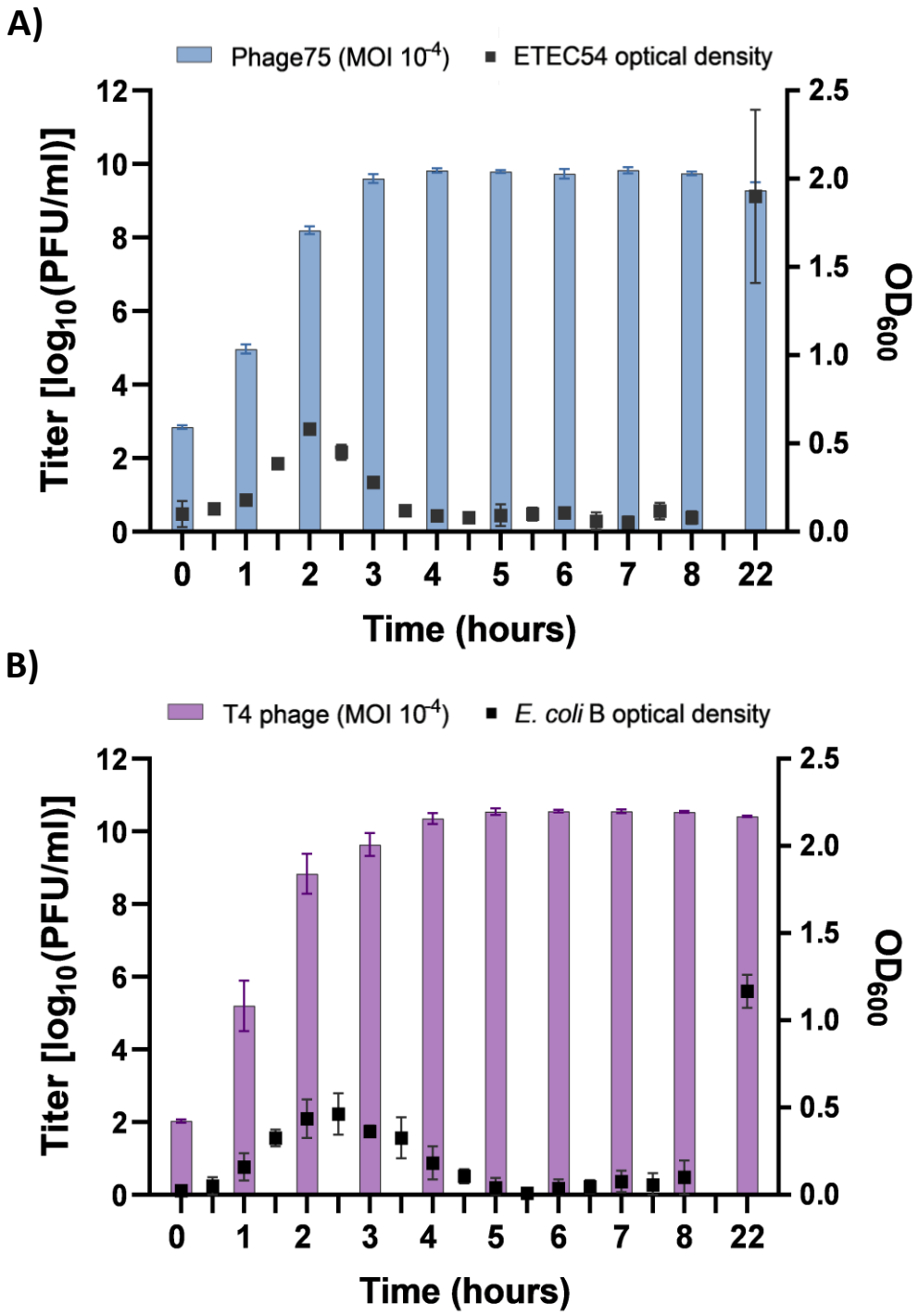


Figure 7. Comparison of phage titer and bacterial optical density (OD) throughout production at 1 L scale in LB medium. Phage and host combinations: **A)** Phage75 and ETEC54; **B)** T4 phage and *E. coli* B. A 1 mL sample was removed from production volume every hour and filter sterilized for titer determination. Additionally, an OD_{600} measurement was taken every 30 minutes (Biochrom WPA CO8000 cell density meter). Error bars show standard deviation from the mean (n = 3).

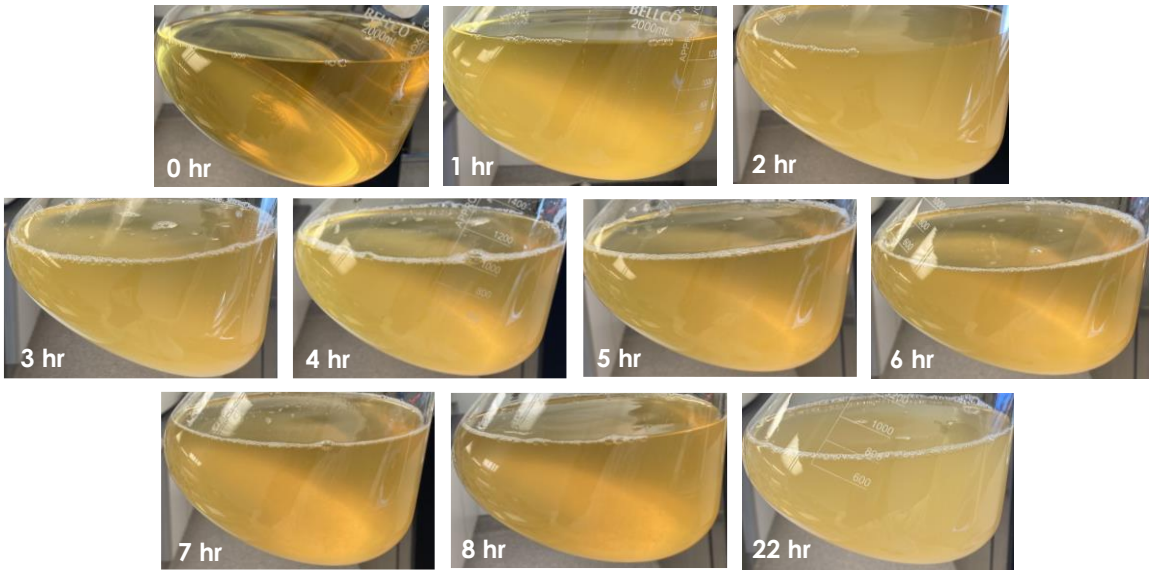


Figure 8. Appearance of 1 L Phage75 production with ETEC54 host in LB medium over time. Production conditions: 4% infection load, 37°C incubation, 120 rpm shaking (Excella® E24 Incubator Shaker).

3.8 Medium-scale production

Productions in LB, APS, and Opt. M9 were carried out in 100 mL to see if comparable titers to those in small-scale (Section 3.6, Figure 5) could be achieved. Based on harvest time investigations in LB (Section 3.7, Figures 6-8) a 4-hour harvest time was chosen to see if this amount of time was suitable for productions carried out in APS or Opt. M9. Overall titers from these productions were 10.10 ± 0.10, 9.89 ± 0.09, and 9.62 ± 0.05 $\log_{10}$(PFU/mL) for LB, APS, and Opt. M9 respectively (Figure 9 B). One-way ANOVA and Tukey multiple comparisons test showed no significant difference between titers achieved in LB or APS, but significance between LB and Opt. M9 ($p = 0.0016$) and between APS and Opt. M9 ($p = 0.0237$). Production flasks were imaged every hour with population-wide lysis seen in LB and APS but not in Opt. M9 (Figure 10).

3.9 Large-scale production

Phage75 production in APS was scaled-up to 1 L to see if overall titer was like that previously achieved in LB (Figure 7 A). The titer at 3 hours was 9.60 ± 0.12 and 9.58 ± 0.09 $\log_{10}$(PFU/mL) for LB and APS respectively, with titers at 4 hours being 9.83 ± 0.06 and 9.65 ± 0.09 $\log_{10}$(PFU/mL) for LB and APS respectively (Figure 9 C). Furthermore, conditions in LB were scaled up for production in the CellMaker bioreactor at a volume of 3 L. Titers obtained from this run were: 9.60 ± 0.06 and 9.74 ± 0.02 $\log_{10}$(PFU/mL) for 3 hours and 4 hours respectively. Additionally, population-wide lysis was observed between 3.5-4 hours with a noticeable reduction in turbidity (Appendix A.4 B and C). The final OD_{600} measured after 4 hours was 0.12; similar to 0.09 ± 0.014 reached at 1 L in shake flasks (Figure 7 A). Furthermore, one-way ANOVA and Tukey multiple comparisons test showed no significance in titer differences among samples from production runs at 1 L in shake flasks or at 3 L in the

CellMaker. A gradual increase in foaming was observed over the first 3 hours of production, with a huge increase in foam during the final hour that completely filled up the bioreactor headspace.

3.10 Production scalability

Over an 8-hour period, Phage75 and T4 phage titers were similar in LB medium at 3 different scales of production: 0.01 L, 0.1 L, and 1 L (Figure 9 A). The titers obtained for Phage75 production were 9.77 ± 0.06, 9.91 ± 0.08, and 9.74 ± 0.05 $\log_{10}$(PFU/mL) for volumes 0.01, 0.1, and 1 L respectively, whereas for T4 phage, the titers were 10.70 ± 0.08, 10.50 ± 0.02 and 10.54 ± 0.03 $\log_{10}$(PFU/mL).

Regardless of whether productions ran over 4 hours or 8 hours, one-way ANOVA and Tukey multiple comparisons test were carried out between all productions in LB, APS, or Opt. M9 media among all scales: 0.01 L, 0.1 L, and 1 L (Figures 5 A, 9 B and C). These tests revealed no significance between titers for productions carried out in APS or LB at any scale and the titer achieved in the CellMaker production ($p > 0.05$). There was also no significance between titers for shake flask productions at 0.1 L and 1 L scales for media APS or LB; $p = 0.1287$ and 0.0855 respectively. Significance was seen between productions in either 0.01 L APS or LB and 0.1 L APS, with values $p = 0.0033$ and $p = 0.0010$ respectively, and between 1 L LB, with values $p = 0.0146$ and 0.0042 respectively. Titers from productions in Opt. M9 at 0.01 L and 0.1 L scales were significant ($p = 0.0004$), with the production at 0.01 L additionally being significant to 1 L APS productions ($p = 0.0007$). However, for other productions carried out in Opt. M9 medium at 0.01 L, no significance ($p > 0.05$) was seen between productions in 0.1 L APS or LB, and 1 L LB. Furthermore, no significance ($p > 0.05$) was seen between production

in 0.1 L Opt. M9 and 0.01 L and 1 L productions in either LB or APS. The titer achieved at any scale in Opt. M9 medium was not significant ($p > 0.05$) to that achieved in LB in the CellMaker.

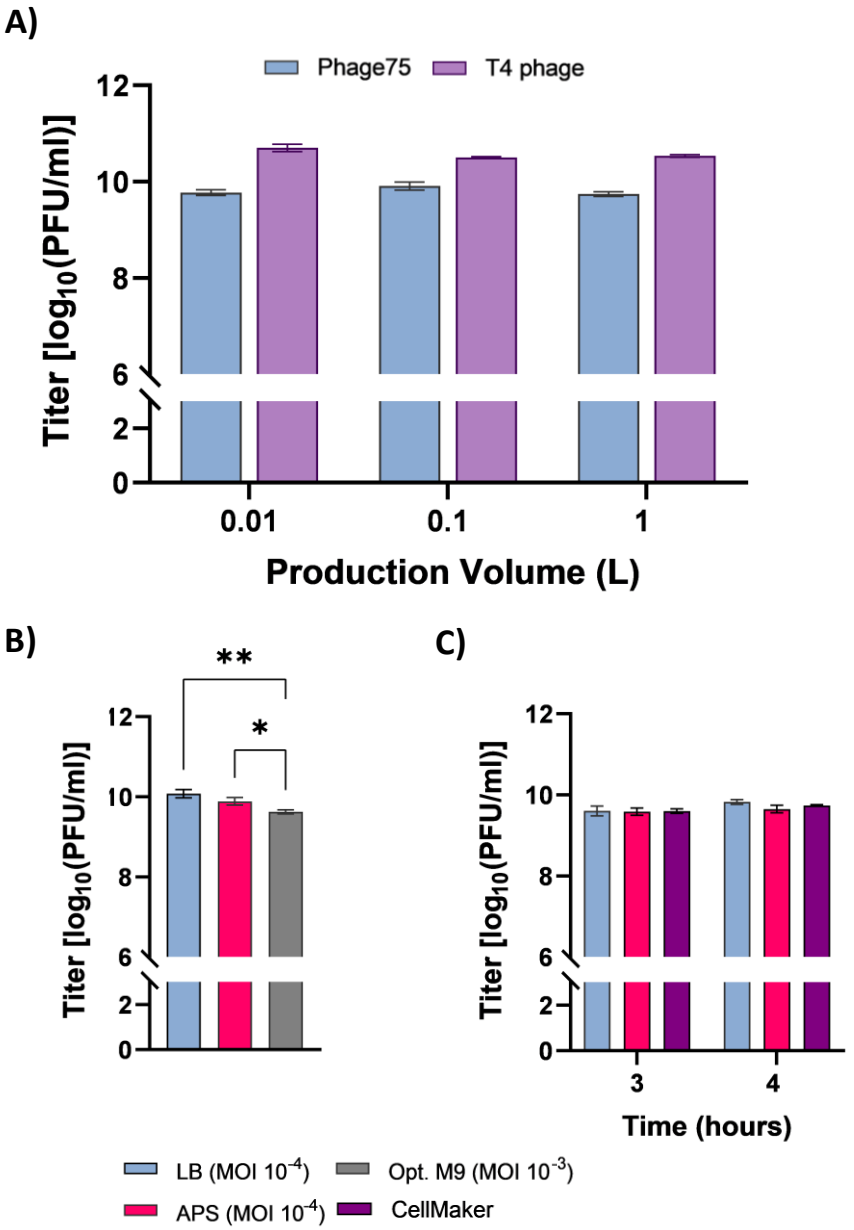


Figure 9. Phage titers for scaled-up productions. All productions had a 4% infection load and a 37°C incubation. **A)** Summary of titer achieved at different production volumes of LB medium after 8 hours**.** Host (phage/MOI): ETEC54 (Phage75/10^{-4}) and *E. coli* B (T4 phage/10^{-4}). **B)** Comparison of media at 100 mL and harvested at 4 hours. **C)** Comparison between 1 L productions and CellMaker production harvested at 3 or 4 hours. CellMaker production carried out in LB with 10^{-4} MOI. Error bars show standard deviation from the mean (n = 3), however for the CellMaker run, n = 1 with error bars rather showing standard deviation among technical replicates. Statistics performed using one-way ANOVA with Tukey multiple comparisons test, ** $p < 0.01$; * $p < 0.1$.

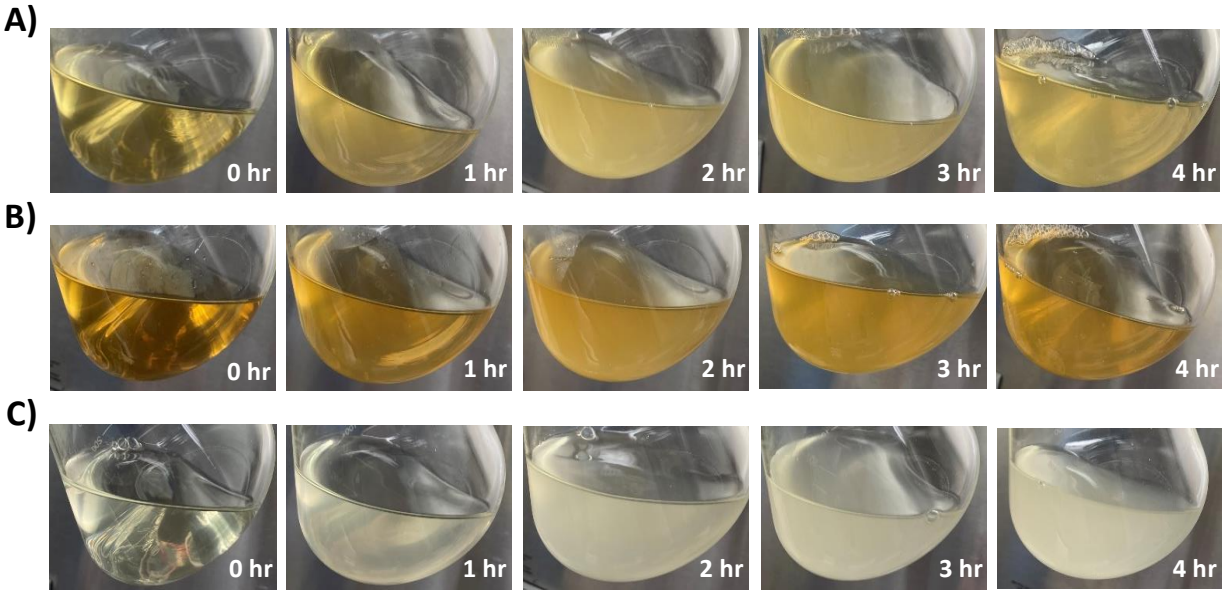


Figure 10. Appearance of 100 mL scale production of Phage75 with ETEC54 host in different media: A) LB; **B)** APS; **C)** Opt. M9. Production conditions: 4% infection load, 37°C incubation, 160 rpm shaking (Excella® E24 Incubator Shaker), harvest time 4 hours.

3.11 Efficiency of Plating (EOP)

Phage75 was plated on 2 additional bacteria: *E. coli* B and *E. coli* C. The EOP of Phage75 was 3.65×10^{-7} on *E. coli* B and 96.50 on *E. coli* C. Plaques were larger with a hazy circumference when plated on *E. coli* C compared to when plated on ETEC54 (Figure 11). No plaques were observed on *E. coli* B at any dilution, except once for a technical replicate of plating at a dilution of 10^0 (Figure 11, Appendix A.5).

3.12 Large-scale production using *E. coli* C as host

Based on Phage75 plaquing ability, *E. coli* C was explored as an alternative host for production. At 1 L in LB, Phage75 titers obtained at 3, 4, and 5 hours were 7.64 ± 0.09, 7.77 ± 0.09, and 7.94 ± 0.01 $\log_{10}$(PFU/mL) respectively (Figure 12 A). Additionally, population-wide lysis did not occur as OD_{600} measurements showed continual *E. coli* C growth despite Phage75 presence and propagation. This was visually observed as the production media remained turbid without ever becoming clear. When Phage75 was rather produced in 1 L APS medium, titers obtained were 7.97 ± 0.31, 8.30 ± 0.43, 8.36 ± 0.46 $\log_{10}$(PFU/mL) for hours 3, 4, and 5 respectively (Figure 12 B). Despite a slightly higher titer produced, population-wide lysis was again not visualized during production (Appendix A.6). For both series of productions, the average difference in titer between the 4- and 5-hour samples was not significant; LB ($p = 0.0749$), APS ($p = 0.9826$).

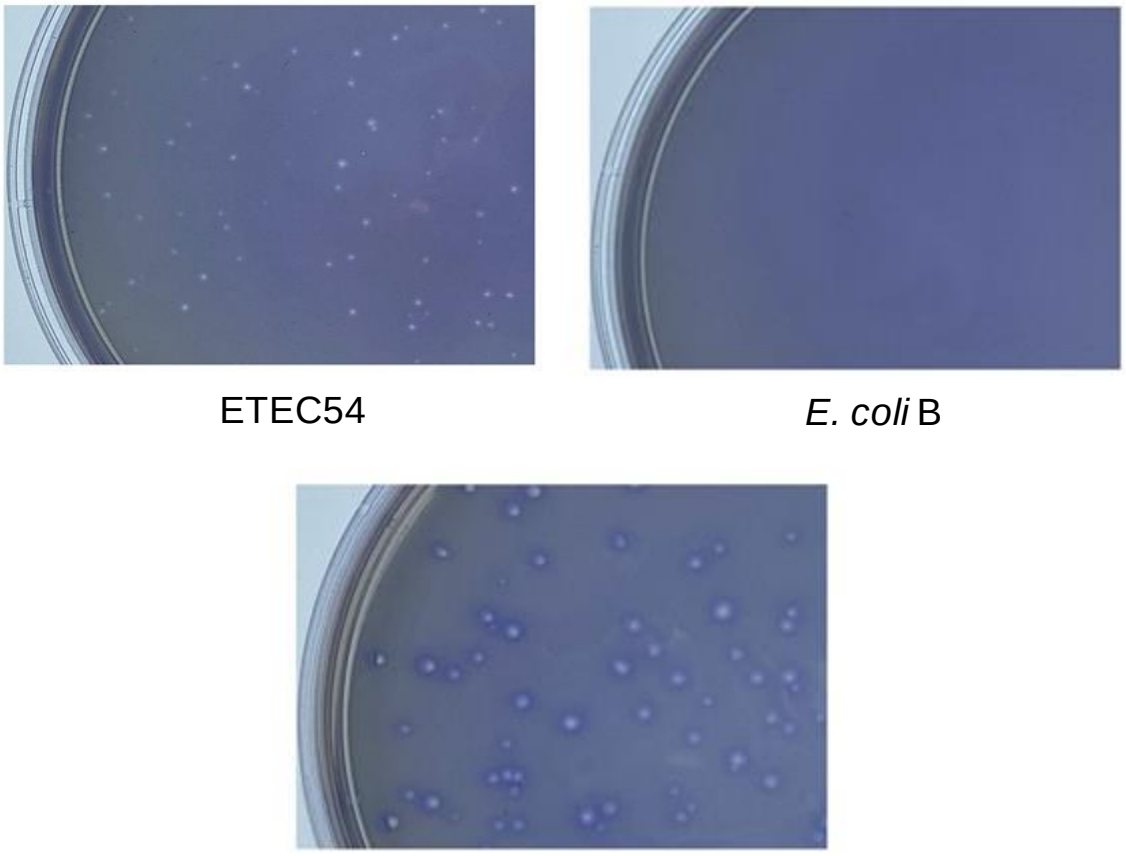


Figure 11. Phage75 plated on different bacterial hosts at 10^{-6} dilution. Overlay: 0.2% agarose in LB. Plates stained with 0.01% crystal violet.

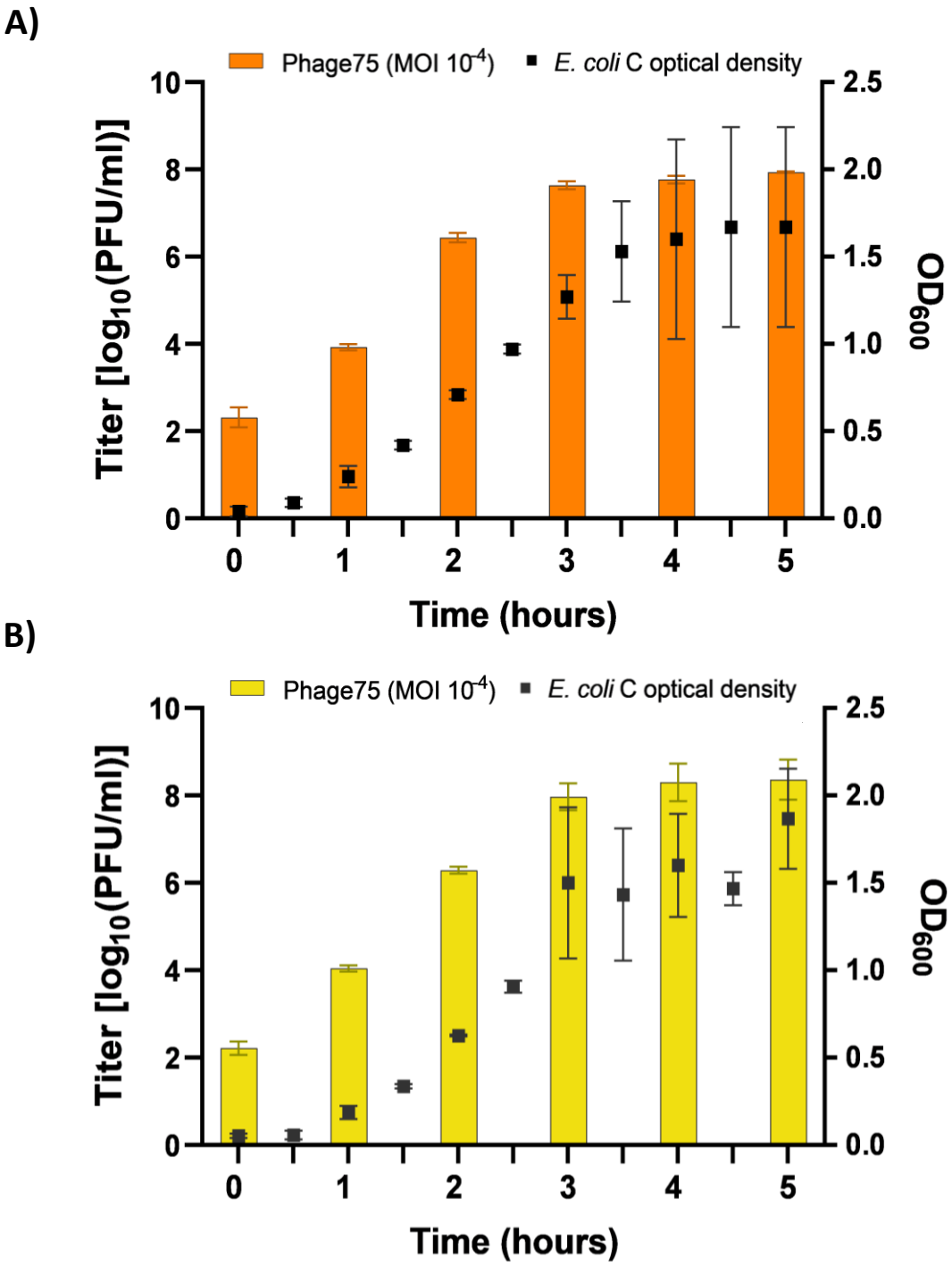


Figure 12. Comparison of Phage75 titer and host optical density (OD) overtime using *E. coli* C. Productions were carried out at 1 L scale in **A)** LB or, **B)** APS. A 1 mL sample was removed from production volume every hour and filter sterilized for titer determination. Additionally, an OD_{600} measurement was taken every 30 minutes (Biochrom WPA CO8000 cell density meter). Error bars show standard deviation from the mean (n = 3).

CHAPTER 4.0: DISCUSSION

Due to the ever-pressing issue of antibiotic resistance against ETEC, alternative strategies for dealing with these infections are needed within the swine industry (Fairbrother et al., 2005). Phages are a promising candidate due to their specificity towards bacteria and the lack of cross-resistance with antibiotics (Loc-Carrillo & Abedon, 2011). However, if phages are to become a viable therapeutic option against ETEC, ways to mass-produce them at a high titer and in an efficient manner need to be established. To help streamline this process, screening assays were developed to investigate multiple variables and conditions in combination for Phage75. The most promising conditions predicted from these assays were carried out at 0.01 L, 0.1 L and 1 L scales to determine phage titer, pursuing ones which yielded the highest titers at larger scales. The optimized production strategy for Phage75 determined in shake flasks was translated to a CellMaker bioreactor to conclude this work and demonstrate an industrial level of phage manufacturing.

High-throughput methods have been used to determine bacterial growth rates (Hall et al., 2014), phage cocktail efficiency (Niu et al., 2021), MOI conditions (S. G. Kim et al., 2021), phage virulence (Storms et al., 2020), and phage host range (Xie et al., 2018), proving just how valuable and useful they are in research. In addition to this, phages have been detected and quantified in similar ways based on degree of host lysis monitored via decreasing OD_{600} (Rajnovic et al., 2019). Because of this, screening assays using 96-well plates were designed to investigate different growth conditions for Phage75 and probe its production overtime. These assays were useful for investigating variables media and MOI (Figure 1), temperature (Figure 4), and harvest time (Figure 1 and 4), but otherwise not overly useful in identifying a benefit of ion supplementation (Figure 3). Specifically, low MOIs and short harvest times were predicted to

yield more phage in nutrient rich media, compared to higher MOIs and longer harvest times in more minimal media. Examples include a 10^{-4} MOI and 4.5-5 hours harvest time when using LB and APS, compared to a 10^{-3} MOI and 6 to 8-hour harvest time when using Opt. M9 medium. Similarly, using a 10^{-4} MOI in LB medium has resulted in high titer for a *Staphylococcus aureus* targeting phage (S. G. Kim et al., 2021). Even though a temperature decrease to 33°C from 37°C in media LB, APS, and Opt. M9 showed a trend towards improved Phage75 production, differences in titer were not statistically significant (Figure 5). Because of this, a temperature of 37°C was chosen to benefit ETEC54 host, because doing so presumably accelerates growth and phage infection cycles, leading to shorter harvest times (Agboluaje & Sauvageau, 2018). Furthermore, screening assays predicted that supplementing $MgSO_4$ and $CaCl_2$ would extend harvest time and that productions may experience incomplete lysis based on OD_{600} readings (Figure 3 C and D). To investigate this more, small-scale productions were carried out with 6 mM $MgSO_4$ supplementation and revealed a titer improvement in LB, but not in APS medium (Figure 5 B). However, since an 8-hour harvest time was chosen for these productions to control the number of variables being tested, it is unknown whether population-wide lysis occurred and optimal harvest time was reached. Ion supplementation was discontinued from further study to possibly enhance production efficiency and because productions in APS medium were not improved by it.

Translating assay conditions to shake flasks was valuable for quantitative comparisons between different growth conditions (Figure 5). These productions revealed complex media to better support Phage75 production and confirmed that the chosen MOI were suitable. Results in screening assays (Figure 1 and 4) and all Phage75 titers in shake flask productions were similar (Figures 5, 9 B and C), whether LB or APS medium was chosen. These results show APS is a

suitable medium to support phage production and can replace LB. APS medium satisfies the requirement that biological drugs need to be produced using animal-free components to meet regulatory guidelines (Regulski et al., 2021) as it contains plant-based soy hydrolysate rather than animal-based tryptones like in LB. In fact, APS medium has been used to prepare a vaccine using 10 L and 300 L bioreactors (Bell et al., 2009) as well as produce phages using petri dishes (Merabishvili et al., 2009) for clinical use. Depending on the phage, using animal protein-free media can also lead to higher titers and shorter harvest times compared to productions carried out in media containing tryptones (Kolenda et al., 2022).

Despite this, defined media is thought to lead to more consistent productions with simpler downstream processing (Stanbury et al., 2017), which is why M9 minimal medium supplemented with glucose was investigated for Phage75 production. A MOI of 1 for Phage75 and ETEC54 was required for cell lysis and OD_{600} reduction of ETEC54 (Figure 1 E) compared to lower MOI like 10^{-3}, for T4 phage and *E. coli* B (Figure 1 F). However, when tryptones were added to M9 minimal medium, designated Opt. M9, ETEC54 grew better and was also lysed at a MOI of 10^{-3} (Figure 1 C), thus reducing the Phage75 input by 1000-fold to achieve a similar outcome. Due to these observations, Phage75 likely requires supplementation with amino acids for its successful infection and propagation. It is difficult to suggest though which amino acids are more important than others, as this would depend on the type of protein being expressed and the level of expression, going beyond the scope of this work and requiring further experimentation and analysis. A much quicker method would be to screen different formulations of M9 minimal media supplemented with different amino acids in a 96-well plate. Trendlines showing lysis at lower MOI would suggest amino acids in those wells benefited Phage75 propagation and further provide insight into medium design for its production. Interestingly,

glycine (S. G. Kim et al., 2021) and tryptophan (Storms et al., 2010) have been shown to impact phage yield in productions for *S. aureus* and *E. coli* phages respectively.

Carbon sources were not investigated in this study, with only glucose supplemented into media when needed since glucose supplemented media yields higher titers for T4-like phages compared to when other carbon sources are used (J. Silva et al., 2021). In contrast, other research has rather shown supplementing glycerol or galactose instead of glucose to increase phage production (S. G. Kim et al., 2021). Screening different carbon sources in conjunction with amino acid supplementation may lead to combinations best suited for Phage75 production and allow creation of a defined medium. While nitrogen sources were also not investigated, removing complex nitrogen sources like peptones, meat extract, and yeast extract from medium has been seen to negatively affect the infection cycle of *Lactobacillus spp.* phages (Marcó et al., 2015; Zaburlin et al., 2017). This suggests that nitrogen sources or different concentrations of nitrogen may have implications in phage production and could additionally be screened in assays.

The ideal time to harvest phage is at population-wide lysis when the medium becomes less turbid and OD_{600} decreases (Agboluaje & Sauvageau, 2018). Screening assays show potential harvest times to vary based on different operating conditions (Figure 1, 3, and 4); however, following drops in OD_{600}, all ETEC54 reduction curves tend to settle in plateau near media blank levels in the presence of Phage75. These observations raised questions as to whether population-wide lysis marks the end of a production, or rather if extending harvest time results in increased titer since productions in LB medium are influenced more by incubation time than in minimal media (J. Silva et al., 2021). To investigate this, 100 mL productions were carried out in LB for 8 hours with samples removed each hour to monitor phage titer. Statistical

analysis showed no significant difference in titers after 4 hours for phage and host combinations Phage75 and ETEC54, as well as for T4 phage and *E. coli* B (Figure 6). Scaled-up experiments at 1 L with OD_{600} measurements every 30 minutes provided evidence peak titer coincided with a drop in OD_{600} for host bacteria around 4 hours for either Phage75 or T4 phage productions (Figure 7). Additionally, any difference in titer between 3 and 4 hours was not statistically significant (Figure 9 C), suggesting production could be shortened by another hour. Population-wide lysis has been seen at these harvest times before using phage-and-host combination T4 phage and *E. coli* B (Sauvageau & Cooper, 2010). Similar studies monitoring lytic phage productions in *S. aureus* overtime show peak titer is not necessarily reached at the same time and is phage- and medium-dependent (Kolenda et al., 2022). Despite this, those studies show similar trends as reported here, with phage titer staying constant for several hours once peak titer is reached, followed by potential decrease upon further overnight incubation. This was seen in the present study as there was no improvement in phage titer in samples taken at 22 hours compared to 4 hours. Planning an efficient phage production strategy should always include harvest time investigations to see if titers achieved earlier in production are similar to those achieved later on (J. Silva et al., 2021).

Another benefit to harvesting a batch production earlier is prolonged susceptibility of host bacteria to phages. It was observed that following a 22-hour production of Phage75, ETEC54 host rebounded in growth and flasks were highly turbid (Figure 8). Samples from these flasks were streaked out for isolated colonies and then tested for susceptibility against Phage75. The results showed that 8/9 colonies were still susceptible to Phage75 stock, however 4/9 colonies were not to the Phage75 samples produced from each respective run, suggesting some level of resistance to phage infection had resulted (Appendix A.3). It is not uncommon for

prolonged exposure of phage with bacterial hosts to result in bacteria developing resistance to phage infection (Luria & Delbrück, 1943). While the present study proposes harvesting production at 4 hours and not 22 hours, this potential resistance from overnight production is not a concern for manufacturing Phage75. However, it does indicate that Phage75 alone may not be sufficient at fully clearing ETEC infections and may be an issue in a therapeutic setting. To help with this, using different ETEC-targeting phages in cocktail with Phage75 and/or the addition of antibiotics may be the best course of action in the future to prevent resistance (Diallo & Dublanchet, 2022).

Variables and conditions known to impact phage production are typically determined in a scalable fashion by factors of 10 (Garcia-Ochoa & Gomez, 2009), which was adopted in the present study by investigating productions in shake flasks at 0.01 L, 0.1 L, and 1 L volumes. In LB medium, no significant difference was seen between titers obtained in 0.01 L and 1 L of medium harvested at 8 hours (Figure 9 A), with a similar result seen in APS medium, even though harvest time varied between these experiments from 8 hours to 4 hours (Figure 5 A and 9 C). Additionally, no significant difference in achieved titers was found between 0.1 L and 1 L productions in either LB or APS, nor between these shake flask productions and the 3 L LB CellMaker production (Figures 9 B and C). These results show conditions in either LB or APS media are robust in supporting Phage75 production and able to translate to larger volumes and even different operating systems, without experiencing a loss in phage titer. A similar outcome was observed for T4 phage when production conditions were translated from shake flasks at 20 mL to a bioreactor at 3 L (Ali et al., 2019). However, for Phage75 and ETEC54, scalable production was not observed in Opt. M9 (Figure 5 A and 9 B), although this may have been influenced by a reduced harvest time at the 0.1 L scale. Regardless, no signs of population-wide

lysis in Opt. M9 were observed throughout the experiment (Figure 10 C). Because developing an efficient process for Phage75 production was priority, Opt. M9 was removed from study, leaving LB and APS.

To avoid undesirable traits associated with a host, such as virulence, multi-drug resistance, or toxin production, surrogate hosts may be preferred to produce phages (García et al., 2019; Gill & Hyman, 2010; Merabishvili et al., 2018). This was investigated by using *E. coli* C rather than ETEC54 to produce Phage75 based on high EOP (Figure 11). Unfortunately, under the same production conditions, *E. coli* C led to a 2.1 $\log_{10}$(PFU/mL) decrease in Phage75 titer after 4 hours in LB medium compared to when ETEC54 was used (Figures 12 A and 9 C). Using APS medium instead did not change this outcome of a reduced titer (Figure 12 B). Similar results where a high EOP did not translate to high production titer were observed in other studies (Kolenda et al., 2022), suggesting this method of host selection may exaggerate future success of productions. Even though employing alternative hosts can lead to decreased phage production, this outcome is often preferred over working with and growing large quantities of multi-drug resistant bacteria (González-Menéndez et al., 2018).

To see if the optimized Phage75 production strategy could be translated to a CellMaker bioreactor, a single, proof-of-concept run was carried out in LB medium. While this production was successful is yielding similar titers to those achieved in shake flasks (Figure 9 C), there were some concerns a higher titer had been sacrificed since the CellMaker was expected to increase phage production in terms of PFU/mL (*Application Note: Phage Amplification for Crop Protection with KU Leuven*, 2020). A possible explanation as to why this wasn't observed could be the excessive foaming present in the final hour of production. Foam was seen to fill the headspace in the bioreactor bag, causing so much pressure to build-up that manual force was

repeatedly applied to encourage foam to leave the bag and enter the condenser liner through the vent tube. A rapid increase in foam is indicative of population-wide lysis and indicates harvest time (Agboluaje & Sauvageau, 2018). However, foam and bubbling can cause stress on phages, possibly leading to their inactivation (Adams, 1948; Yu et al., 2010) and consequently, result in a reduced titer in production. One study also speculated their decreased filamentous phage production with *E. coli* K91 was a result of foaming (Grieco et al., 2009). A low airflow to provide mixing was selected to mitigate inevitable foaming; however, doing so may have negatively affected ETEC54 growth, which could also reduce the phage titer. For these reasons, it is recommended to use sufficient airflow and add an antifoaming agent to reduce foaming for future CellMaker productions. Antifoaming agents work at the surface to dissipate foam and must be added in small quantities to avoid affecting the OTR during a production (Stanbury et al., 2017). Many different types of antifoams are available, including silicones, sulfonates, alcohols, and fatty acids such as olive oil (Junker, 2007; Stanbury et al., 2017). Olive oil has been used to mitigate the stress from bubbling on phages and *E. coli* and *Serratia marcescens* host strains (Yu et al., 2010). Despite this, future productions for Phage75 could utilize Antifoam C, an aqueous, silicone-containing antifoam, since Cytophage already has it in stock.

The degree of agitation affects the level of aeration in production and should be maintained in scale-up to avoid negative impacts on phage titer (Agboluaje & Sauvageau, 2018). In the present study, agitation was kept constant at each scale of production, though the effects of agitation and aeration were otherwise not explored as variables. Different agitation rates may be preferential depending on the phage and host system (Ali et al., 2019), production volume (Warner et al., 2014), and operating system used (Bourdin et al., 2014; Sauvageau & Cooper, 2010), but may otherwise not have any impact (González-Menéndez et al., 2018). However,

using lower agitation rates may aid phage infection by preserving tails for adsorption, and can be off-set by manipulating MOI to ensure phages still come into contact with host cells (J. Silva et al., 2021). If agitation is too low though, bacterial cultures will not be sufficiently aerated and may not grow (Grieco et al., 2009), which could negatively impact phage titer. Therefore, further investigations to optimize host ETEC54 growth via enhanced oxygen transfer rate and agitation may be beneficial (Bates et al., 2011).

Phages on average are stable within the range of pH 6 to 8, although depending on the phage, stability is also possible at more acidic or basic conditions (Jończyk et al., 2011). Adjusting the pH to be more acidic, such as pH 5 or 6, has led to an increase or decrease in burst sizes for different phages after host infection (Zaburlin et al., 2017). However, for a filamentous phage with *E. coli* K91 host, maintaining near neutral pH throughout production resulted in higher specific productivity of phage per bacterium, as well as higher overall yield (Grieco et al., 2009). Bacterial metabolism and growth can also be influenced by pH, and can be negatively impacted even by a one unit change (Jin & Kirk, 2018) and could have implications on phage propagation. Therefore, even if lysis is possible for T4 phage at pH ranging from 4-10 (Taj et al., 2014), considerations of how to optimize infection and maximize titer need to be considered for Phage75. Future screening assays exploring different pH for Phage75 production could lead to improvements in titer by ensuring optimal pH for ETEC54 is reached.

CHAPTER 5.0: CONCLUSION

A scalable production method resulting in high titers of Phage75 was successfully defined. High-throughput screening assays allowed predictions as to which growth conditions might lead to high titers in production. Conditions predicted to be the best were then translated to shake flasks to determine titers, which allowed comparisons to be made among conditions and further identified those which impacted phage production the most. Several variables were considered for optimization with MOI, media composition, harvest time, and chosen host appearing to have the greatest impact on production yields. An intermittent MOI of 10^{-4} and short harvest time, 4 hours, were chosen to produce Phage75, with data showing an extended production was unnecessary once peak titer had been reached. This result maximized production efficiency by allowing for shorter batches. Furthermore, using complex media such as LB or APS was better for Phage75 production than minimal media; however future work looking at supplementing in specific amino acids may prove to be beneficial in establishing a minimal defined medium with similar outcomes. Additionally, using a surrogate host like *E. coli* C is possible, but a reduction in titer should be expected. Overall, optimizing phage production in a scalable way and investigating variables seen to affect phage production allows for the development of a robust process that can serve as a framework and provide insight to produce other phages in the future. Therefore, the work presented here is a benefit to subsequent studies investigating phage production, especially when scalability and high efficiency are desired.

Appendix

Figure A.1. Transmission electron micrographs showing morphology of A) Phage75, and **B)** T4 phage.

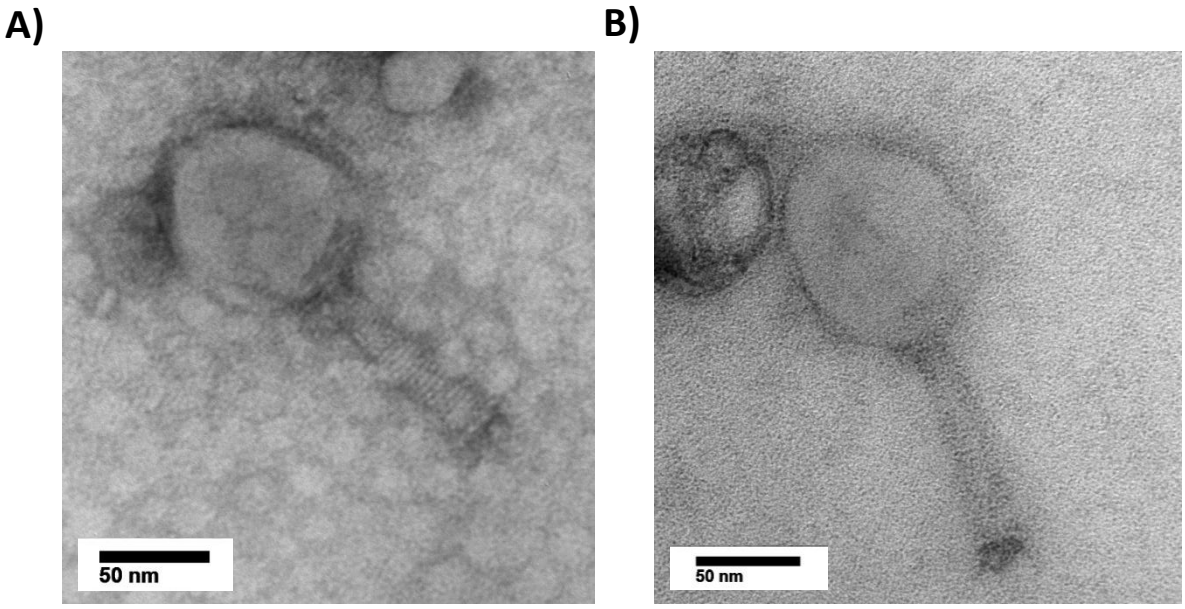


Figure A.2. Titer comparison between different MOI conditions and infection loads for T4 phage production in *E. coli* B host. Productions were carried out in 10 mL of LB incubated at 37°C with shaking at 200 rpm (VWR® Incubating Mini Shaker) and harvested after 8 hours. Error bars show standard deviation from the mean (n = 3).

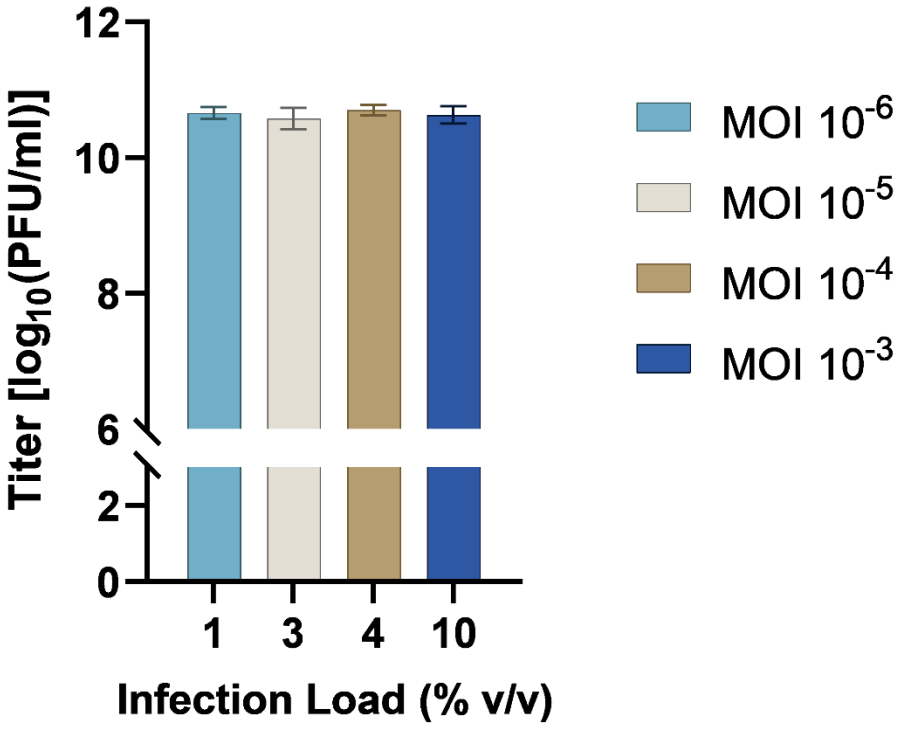


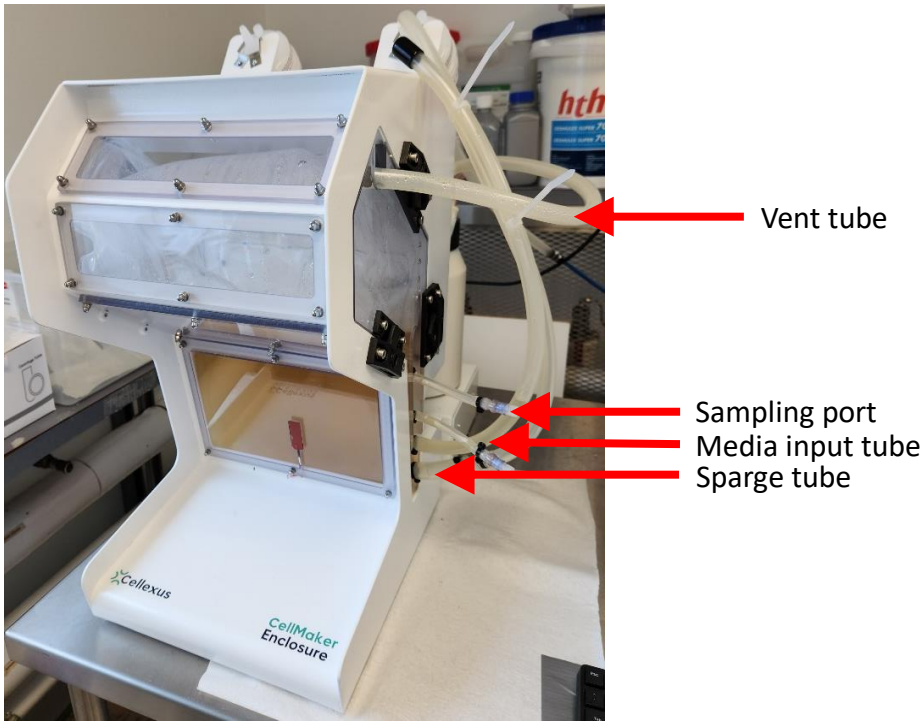
Figure A.3. Phage75 on residual ETEC54 from 22-hour production to check for susceptibility. Phage75 was spot plated at dilutions 10^{-6} and 10^{-7}.

= visible plaques
= no plaques

	Replicate 1		Replicate 2		Replicate 3	
Colony	Sample	Stock	Sample	Stock	Sample	Stock
1						
2						
3						

Figure A.4. CellMaker production run. A) Diagram of CellMaker while in operation; **B)** Appearance of LB at specified timepoints; **C)** Comparison of 4-hour sample to fresh LB medium.

A)



B)



C)

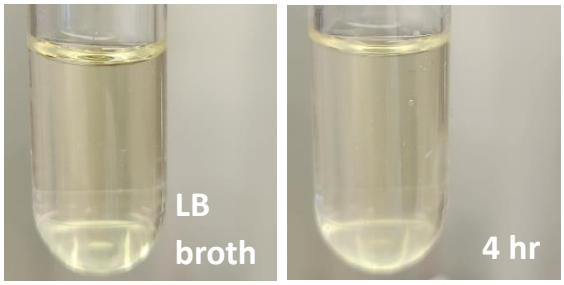


Figure A.5. Phage75 spot plated on *E. coli* B bacterial lawn at dilutions: 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}. Plate was stained with 0.01% crystal violet solution.

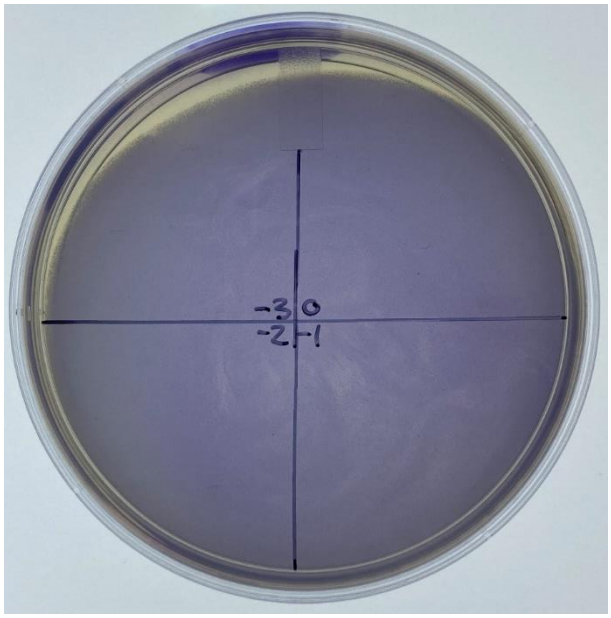
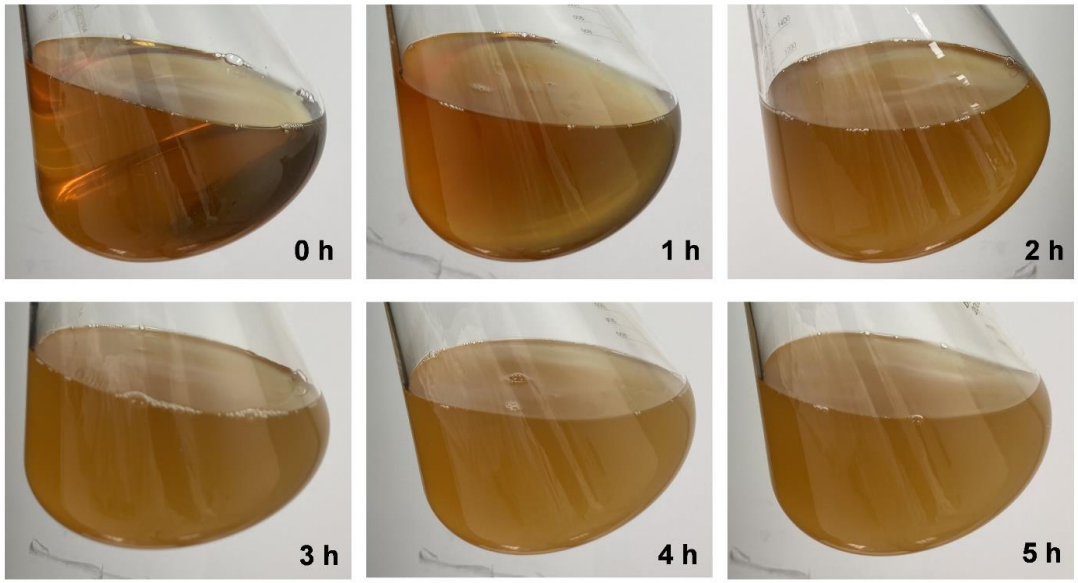
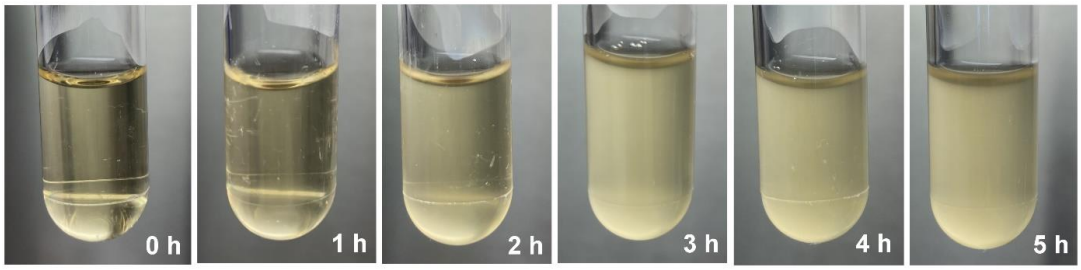


Figure A.6. Appearance of 1 L scale Phage75 production with *E. coli* C host in APS medium. A) 1 L flask volume; **B)** 3 mL aliquot from 1 L flask volume. Production conditions: 4% infection load, 37°C incubation, 120 rpm shaking (Excella® E24 Incubator Shaker).

A)



B)



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