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Optimization of the large-scale production for *Erwinia amylovora* bacteriophages

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Abstract

Background Fire blight, caused by *Erwinia amylovora*, poses a significant threat to global agriculture, with antibiotic-resistant strains necessitating alternative solutions such as phage therapy. Scaling phage therapy to an industrial level requires efficient mass-production methods, particularly in optimizing the seed culture process. In this study, we investigated large-scale *E. amylovora* phage production by optimizing media supplementation and fermenter conditions, focusing on minimizing seed phages and pathogenic strains to reduce risks and improve the seed culture process.

Results We optimized the phage inoculum concentrations and media supplements to achieve higher phage yields comparable to or exceeding conventional methods. Laboratory-scale validation and refinement for fermenter-scale production allowed us to reduce bacterial and phage inoculum levels to 10^5 CFU/mL and 10^3 PFU/mL, respectively. Using fructose and sucrose supplements, the yields were comparable to conventional methods that use 10^8 CFU/mL host bacteria and 10^7 PFU/mL phages. Further pH adjustments in the fermenter increased yields by 16–303% across all phages tested.

Conclusions We demonstrated the successful optimization and scale-up of *E. amylovora* phage production, emphasizing the potential for industrial bioprocessing with the reduced use of host cells and phage seeds. Overall, by refining key production parameters, we established a robust and scalable method for enhancing phage production efficiency.

Keywords Phage production, Large-scale, Fire blight, *Erwinia amylovora*, Optimization

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Background

Erwinia amylovora persists as a global agricultural threat that spreads rapidly and poses significant risks, and it has recently been recognized as an invasive pathogen in East Asia [1]. The concurrent development of -resistance to antibiotic compounds is a concern that could potentially harm agricultural productivity and economic stability [2, 3]. In terms of addressing this concern, the One Health approach is essential for recognizing the interconnectedness of human, animal, and environmental health [4]. By integrating best practices in farming and environmental management, One Health promotes sustainable agriculture and helps mitigate antibiotic resistance [5]. Coordinated efforts among farmers, veterinarians, policymakers, and public health professionals support alternative disease management strategies, such as improved hygiene, biosecurity measures, and the use of biocontrol agents [6, 7].

Phage therapy has emerged as a promising alternative for effective pathogen control in agriculture [8–10]. Among the emerging alternative treatments, such as microbiome therapies, phage therapy has shown particular promise. Unlike its medical applications, where phage therapy often requires remarkably low doses per administration, significantly larger quantities are required for agricultural purposes considering the conventional practices in crop and fruit cultivation. Therefore, it is crucial to recognize the substantial disparities in phage quantities required between the medical and agricultural contexts. For instance, AgriPhage recommends a concentration of 9.46×10^{12} PFU/mL per acre, underscoring the considerable volume of phages necessary for extensive orchards [11]. Given the varying sizes of orchards, ranging from as little as 29 acres to as extensive as 49,535 acres, the magnitude of this requirement becomes apparent. This pronounced contrast underscores the unique challenges and requirements inherent to the implementation of phage therapy in agricultural contexts.

Advanced mass-production technologies using fermenters are essential to meet the demand for large quantities of phage therapy in agricultural applications [12]. Numerous studies have highlighted the utility of fermenters in bacteriophage production, enabling various applications, such as optimizing nutritional factors and cultivation parameters, as well as implementing batch culture, continuous culture, and culture-independent real-time phage titer-checking systems [13]. Response surface methodology (RSM), a powerful tool for process optimization, has demonstrated significant explanatory power in enhancing phage production processes [14–16]. Additionally, advancements in bioreactor design and automation have facilitated precise control of growth conditions, ensuring consistent and scalable phage production [17, 18]. The use of fermenters in phage production aligns well with regulatory and licensing requirements, as these systems allow for controlled and standardized production processes, which are crucial for ensuring product safety and efficacy in agricultural settings [19].

In this study, we focused on optimizing the mass production of *E. amylovora* bacteriophages. Using RSM, our goal was to determine the optimal conditions for efficient and cost-effective large-scale phage production. By prioritizing the optimization of production parameters, this study addresses the threats posed by fire blight, offering an affordable strategy for mass-producing *E. amylovora* phages.

Methods

Microorganisms and culture conditions

We used *E. amylovora* TS3128, the sole reference strain authorized for research purposes in South Korea. The pEa_SNUABM_8 (pEa_8), pEa_SNUABM_27 (pEa_27), pEa_SNUABM_31 (pEa_31), and pEa_SNUABM_47 (pEa_47) phages, which were previously isolated from a river adjacent to a diseased orchard, were employed [20, 21]. The Fifi318 and Fifi451 phages were provided courtesy of Dr. Eunjung Roh of the Rural Development Administration of Korea (Jeonju, South Korea) [22]. The pEa8, pEa27, and pEa31 phages were used to identify the optimal conditions for phage production, while pEa_27, pEa_47, Fifi318, and Fifi451 were used to validate the optimized phage production process. For the cultivation of microorganisms, both nutrient agar and nutrient broth (NB) media supplemented with 0.4% agar as the top layer were used for phage propagation at a constant temperature of 27 °C over a period of 18–24 h. Bacterial cultures incubated overnight (approximately 18 h) were used to establish bacterial lawns, and serial dilutions performed using SM buffer (50 mM Tris, pH 7.5; 100 mM NaCl; 10 mM $MgSO_4$). After incubating at 27 °C overnight, the plaques were counted.

Phage propagation and purification

Phage amplification was performed using the double-layer agar method, as previously described [23]. The phages were harvested by collecting the top agar layer in an SM buffer solution, before thoroughly mixing for 1 h to ensure complete dissociation of phages from the agar. Subsequently, the phage lysates were centrifuged at 12,000 × *g* for 10 min, and the supernatant was precipitated using a solution of 10% (w/v) polyethylene glycol and 0.5 M sodium chloride. Purification was achieved through cesium chloride density gradient centrifugation. The samples were ultracentrifuged at 182,000 × *g* for 3 h in a Type 70 Ti fixed-angle titanium rotor. Distinct bands of phage particles were carefully extracted from the gradient and dialyzed using Slide-A-Lyzer™ Dialysis Cassettes with a molecular weight cutoff of 10,000 to

eliminate residual impurities. The resulting phage preparations, with concentrations confirmed to exceed 10^{10} plaque-forming units (PFU)/mL, were filtered to ensure sterility and stored at 4 °C pending further analysis. We used the 0.45-μm syringe filter to exclude bacterial cells in the lysate while allowing jumbo phages (i.e. pEa_8, pEa_31, pEa_47) to pass.

Effect of the inoculum concentration on phage production

The effects of different concentrations of phage inoculum on phage production were investigated. The following three distinct phages with different characteristics were used: pEa_8, pEa_27, and pEa_31. Initially, an overnight bacterial culture was prepared in 50 mL NB and subsequently adjusted to a concentration of 2×10^5 colony-forming units (CFU)/mL. The phages were then inoculated at concentrations ranging from 3 to 7 Log PFU/mL. Phage amplification was assessed by quantifying the PFUs using the double-layer agar method.

Optimization of nutritional sources for phage production

We employed the one-factor-at-a-time method, which is commonly used in conventional scale-up processes, to identify the optimal nutritional sources essential for phage production [14]. Optimization was tested using the three phages, pEa_8, pEa_27, and pEa_31. Bacteria cultured overnight were prepared in 50 mL NB and subsequently adjusted to a concentration of 2×10^5 CFU/mL. The phages were inoculated at a concentration of 2×10^3 PFU/mL to achieve an MOI of 0.01, which was previously determined to yield the highest production rate. To optimize the carbon source, the basal medium was individually supplemented with 0.5% of fructose, glycerol, glucose, sorbitol, or sucrose. Additionally, the effect of divalent cations on bacteriophage production was explored by supplementing the NB medium with 0.01 M of $CaCl_2$ or $MgCl_2$. To the end, the stability of phages against media supplements, pH, and temperature was confirmed as previous description [24, 25]. Shortly, phages (2×10^5 PFU/mL) were incubated in SM buffer containing each agents, or pH adjusted to 5–8. For the thermal stability phages were incubated in 27 °C and 4 °C served as a control. After incubation (24 h), concentration of phages were assessed in triplicate ($n=3$) using the double-layer agar method to determine any effects on infectivity of phages. The supplement concentrations used were based on the critical concentrations described in previous studies [14].

Design of experiments for phage production optimization

Using the central composite design (CCD) of RSM, an experimental design was developed to establish optimal levels of the selected variables, with Minitab software (v16.2) facilitating the implementation. The optimal concentrations for effective phage production were determined using the three phages, pEa_8, pEa_27, and pEa_31. This experiment was conducted by selecting two substances that exhibited the most optimal effectiveness for phage production. The experimental design encompassed 39 distinct evaluations (Table 1), each replicated thrice. The following quadratic polynomial model was applied to analyze the phage output:

$$Y = b_0 + \sum b_i X_i + \sum b_{ii} {X_i}^2 + \sum b_{ij} X_i X_j$$

where Y represents the predicted outcome, b_0 is the constant term, b_i is the linear coefficient, b_{ii} is the quadratic coefficient, b_{ij} is the interaction coefficient, X_i is the independent variable, ${X_i}^2$ is the squared term, and X_iX_j is the interaction between variables. The model was visualized using contour plots and response surface curves generated for each variable using Minitab (v16.2). The relationship between μ and yield was examined through regression analysis in the same software. The statistical validity of the model was assessed with analysis of variance (ANOVA), considering $p<0.05$ as statistically significant.

Validation of optimized conditions at the flask scale

This experiment was conducted to validate the optimization of two specific parameters, including the phage concentration and the combined effect of culture supplements. Validation was performed using the following four phages: pEa_27, pEa_47, Fifi318, and Fifi451. In the control group, bacterial cultures in the exponential phase (2×10^8 CFU/mL) were used, with phage concentrations set at 2×10^7 PFU/mL and an MOI of 0.1, adhering to the conventional method (CM). Enhanced phage production under optimized conditions was validated by adjusting the bacterial cultures to a concentration of 2×10^5 CFU/mL and setting the phage concentration to 2×10^3 PFU/mL to achieve an MOI of 0.01. The group used to validate the optimal conditions was tested using two setups: one with the optimized phage concentration alone (phage inoculum optimization; IO) and the other with both the optimized phage concentration and additional supplements (phage inoculum and supplement optimization; ISO). The experiments were conducted in flasks and incubated at 27 °C with agitation at 150 rpm for 18 h. After incubation, the phage titers were assessed using the double-layer agar method.

Optimization of phage production in a fermenter

For mass production of phages, the optimized conditions were scaled up to a 5 L fermenter A (KF-5L; Kobiotech, Incheon, South Korea) containing with 2 L NB. Before each operation, all probes (pH meter and dissolved

Table 1 Central composite design (CCD) and experimental design, along with the response for the production of *Erwinia* phage pEa_SNUABM_8, pEa_SNUABM_27, and pEa_SNUABM_31

Standard order	Variables (coded value)		Experimental yield (%)		
	Fructose (A)	Sucrose (B)	pEa_SNUABM_8	pEa_SNUABM_27	pEa_SNUABM_31
1	1.414214	0	265.5172	325.5319	571.4286
2	1	1	282.7586	319.1489	714.2857
3	−1	1	393.1034	346.8085	614.9068
4	−1.41421	0	531.0345	393.617	524.8447
5	0	−1.41421	675.8621	295.7447	428.5714
6	0	0	437.931	336.1702	565.2174
7	0	1.414214	296.5517	348.9362	726.7081
8	−1	−1	472.4138	325.5319	565.2174
9	1	−1	424.1379	278.7234	993.7888
10	0	0	427.5862	338.2979	559.0062
11	0	0	234.4828	489.3617	552.795
12	0	0	462.069	317.0213	500
13	0	0	420.6897	293.617	559.0062
14	1.414214	0	220.6897	314.8936	677.0186
15	0	0	400	306.383	549.6894
16	0	0	413.7931	329.7872	590.0621
17	−1	−1	462.069	276.5957	440.9938
18	0	0	437.931	323.4043	583.8509
19	0	1.414214	213.7931	363.8298	835.4037
20	−1	1	531.0345	312.766	614.9068
21	−1.41421	0	544.8276	357.4468	496.8944
22	1	−1	431.0345	112.766	913.0435
23	0	0	458.6207	312.766	574.5342
24	0	−1.41421	717.2414	289.3617	540.3727
25	0	0	451.7241	342.5532	518.6335
26	1	1	324.1379	312.766	857.1429
27	0	0	420.6897	312.766	593.1677
28	−1	1	455.1724	319.1489	658.3851
29	−1	−1	403.4483	325.5319	534.1615
30	0	0	455.1724	338.2979	543.4783
31	0	0	465.5172	355.3191	611.8012
32	1	−1	451.7241	168.0851	1130.435
33	1.414214	0	282.7586	317.0213	614.9068
34	0	0	472.4138	351.0638	512.4224
35	0	−1.41421	631.0345	291.4894	478.2609
36	0	0	427.5862	314.8936	552.795
37	0	1.414214	272.4138	300	708.0745
38	1	1	251.7241	295.7447	757.764
39	−1.41421	0	562.069	370.2128	496.8944

oxygen (DO) meter) were calibrated and sterilized by autoclaving along with the pH control solutions (1 M HCl and 1 M NaOH). To prevent cross-contamination, only a single phage was used per operation. Consistent conditions were maintained at 27 °C with an agitation speed of 150 rpm. Additional conditions, such as pH levels and the need for oxygen, were evaluated by adjusting the pH to 5, 6, 7, and 8 using sterile 1 M HCl and 1 M NaOH solutions administered through the fermenter. The dissolved oxygen level was maintained at 0.5 kgf/cm²G. Filtered nutrient supplements were added to the medium and mixed thoroughly. The bacterial culture was adjusted to a concentration of 2×10^5 CFU/mL, and after a 30-min adaptation period, phages were introduced at a concentration of 2×10^3 PFU/mL to achieve an MOI of 0.01. Following an 18-h incubation, samples were collected using a syringe, filtered, and the phage titers were measured using the double-layer agar method.

Validation of fermenter-scale phage production

The feasibility of mass production at the fermenter scale was evaluated for four phages: pEa_27, pEa_47, Fifi318,

and Fifi451. The control group had no additional conditions, whereas the optimized group maintained the previously optimized conditions of pH 6 in the fermenter. Bacterial cultures were inoculated at a concentration of 2×10^5 CFU/mL, and phages were introduced at a concentration of 2×10^3 PFU/mL to achieve an MOI of 0.01. The cultures were incubated at 27 °C with agitation at 150 rpm for 18 h. After incubation, the samples were collected, filtered, and the phage titers were measured using the double-layer agar method.

Statistical analysis

Statistical differences were evaluated with Sigmaplot 12.5 (Systat Software Inc., Evanston, IL, USA) using ANOVA, accompanied by the Holm–Sidak test, with statistical significance determined at $p<0.001$. A CCD method within RSM was employed to determine the optimal levels of the selected variables using Minitab 22 statistical analysis software.

Results

Effect of the inoculum concentration on phage production

The effect of the phage inoculum concentration on phage amplification is illustrated in Fig. 1. Experiments were conducted using three model phages (pEa_8, pEa_27, and pEa_31) to analyze the impact that various inoculum levels has on the phage production yield. For pEa_8, the production yield increased from a minimum of 57% to a maximum of 999.66% as the inoculum concentration of phage decreased (Fig. 1a). The pEa_27 phage showed increased production yields of 208% at 5 Log PFU/mL and 201% at 3 Log PFU/mL compared to that of the control (Fig. 1b). The pEa_31 phage demonstrated a significant increase in production yield of 59.72% at 6 Log PFU/mL, 213.86% at 5 Log PFU/mL, 587.31% at 4 Log PFU/mL, and 1,206.99% at 3 Log PFU/mL (Fig. 1c). The results confirmed that all phages exhibited an increase in production yield at inoculum concentrations ranging from 3 to 6 Log PFU/mL of the initial phage inoculum ($p<0.05$). Notably, the highest phage production yield was observed at the lowest inoculum concentration (3 Log PFU/mL). Therefore, this concentration was used in subsequent experiments.

Effect of media supplements on phage production

The effect of media supplements on phage production was evaluated using a bacterial concentration of 2×10^5 CFU/mL and phage inoculum with a concentration of 2×10^3 PFU/mL, which yielded optimal production. This evaluation involved three model phages (pEa_8, pEa_27, and pEa_31). Various carbon and cation sources were individually added to NB medium using the one-factor-at-a-time method. The tested carbon sources included glycerol, glucose, fructose, sucrose, and sorbitol. All phages showed increases in phage production with the addition of supplements (Fig. 2). Additionally, for pEa_8, significant increases were observed after the addition of fructose (34.26%), sucrose (166.29%), and sorbitol (79.77%) (Fig. 2a). The pEa_27 model phage showed substantial increases in phage production with the addition of fructose, glycerol, sorbitol, and sucrose, with fructose and sucrose causing the greatest increases at 432.11% and 3,166.05%, respectively (Fig. 2b). Similarly, pEa_31 exhibited significant increases in production after the addition of fructose (117.94%), sorbitol (95.51%), and sucrose (105.12%) (Fig. 2c). As all supplements resulted in increased phage yields, and fructose and sucrose demonstrated the most substantial effects across all phages, these two supplements were selected for further experiments.

The effects of the two cations, $CaCl_2$ and $MgCl_2$, on phage production were also investigated. For pEa_8, both cations significantly reduced phage production by approximately 99% (Fig. 2d). Compared to the control, the phages, pEa_27 and pEa_31, showed decreases in phage production of 71% and 15%, respectively, after $CaCl_2$ exposure (Fig. 2e and f). Conversely, the addition of $MgCl_2$ increased phage production by 201% for

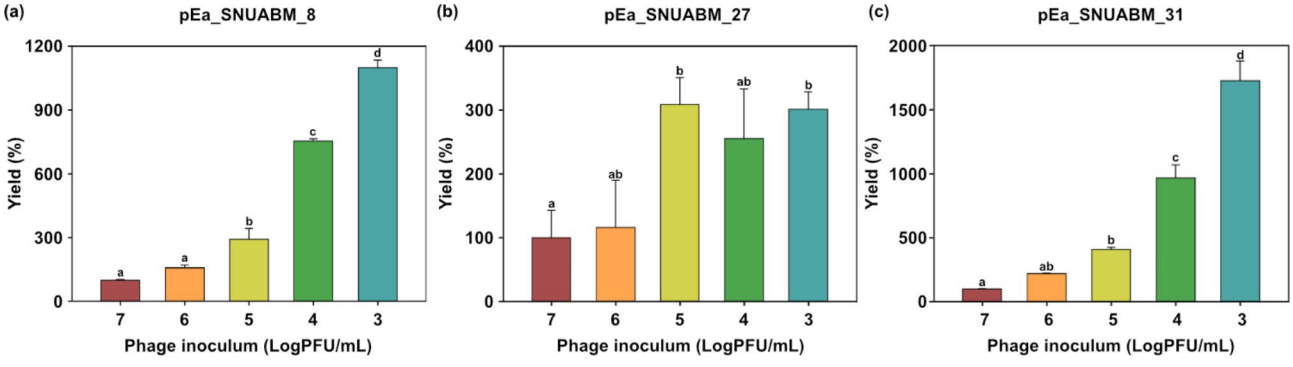

Fig. 1 Phage production yields for *Erwinia amylovora* phages at different inoculum concentrations. Yields for the following *E. amylovora* phages were measured: pEa_SNUABM_8 (**a**), pEa_SNUABM_27 (**b**), and pEa_SNUABM_31 (**c**). Experiments were performed in triplicate. Statistical significance was determined using the Holm–Sidak test, with different letters above the bars indicating statistically significant differences between groups ($p<0.05$)

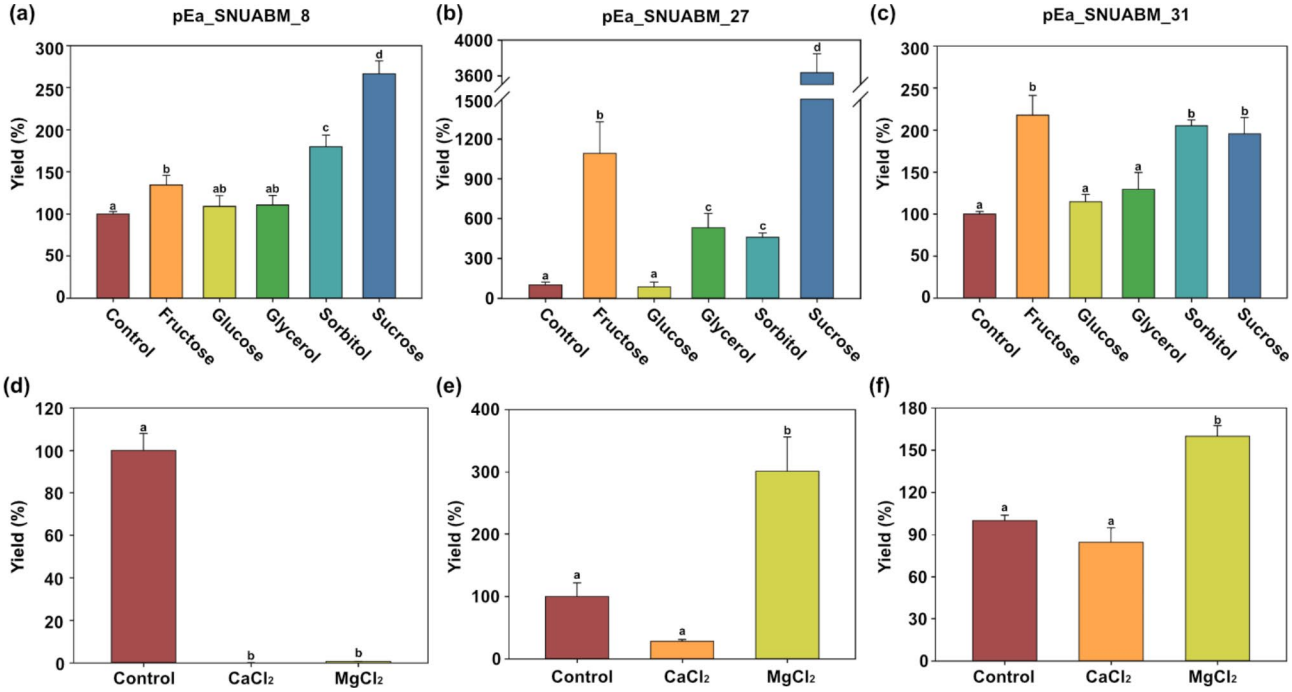

Fig. 2 Screening of media supplements to maximize the production of *Erwinia amylovora* phages. Various carbon sources (**a**, **b**, **c**) and cations (**d**, **e**, **f**) were used, and the following *E. amylovora* phages were tested: pEa_SNUABM_8 (**a**, **d**), pEa_SNUABM_27 (**b**, **e**), and pEa_SNUABM_31 (**c**, **f**). Statistical significance was assessed using the Holm–Sidak test, with different letters above the bars representing statistically significant differences between groups ($p < 0.05$)

pEa_27 and 60% for pEa_31. Despite the positive effects observed for $MgCl_2$, the negative impact that cations had on phage production in one phage led to their exclusion from further studies.

Response surface methodology-based optimization of media supplements for phage yield

We next investigated the effects of selected media supplements on the production of the model phages, pEa_8, pEa_27, and pEa_31, using CCD-based RSM. ANOVA was performed to establish the relationship between phage yield and fructose and sucrose concentrations. The significance of the models was confirmed by measuring p-values < 0.05 for all three model phages, along with high F-values of 13.41, 5.76, and 7.77, respectively. Additionally, the p-values for the lack of fit for all three phages were greater than 0.05, indicating that the models fit the data well (Table 2).

Figure 3 depicts a 2D contour plot of the optimal factor ranges for the maximum phage yield response. For pEa_8, the addition of fructose had positive effects at all levels, whereas the addition of sucrose resulted in higher production at lower coded levels. For pEa_27, lower fructose and higher sucrose levels were associated with a higher phage yield. The pEa_31 model exhibited higher phage yields with increasing concentrations of both supplements. Overlapping optimal concentration ranges for these phages were used in subsequent experiments (Fig. 3d). For pEa_8, the phage yield was higher ($>10^9$ PFU/mL) when lower concentrations of fructose and sucrose were added (Fig. 3a). In contrast, for pEa_27, fructose showed an inverse correlation with production yield, whereas sucrose exhibited no significant dependence (Fig. 3b). For pEa_31, both fructose and sucrose concentrations positively correlated with phage yield (Fig. 3c). When combined, the optimal concentrations for the highest phage production were identified (marked in red in Fig. 3d), with coded values of −0.5 for fructose and −1.0 for sucrose selected for media supplements.

Validation of optimized phage production conditions

For the supplementary group the effects of fructose and sucrose was evaluated, in combination to the optimized phage concentration. Using the conventional method (CM), pEa_27 achieved a yield of 9.46 Log PFU/mL (Fig. 4). Under optimized conditions, the yield of inoculum optimization (IO) was 7.88 Log PFU/mL, and the addition of supplements increased the inoculum and supplement optimization (ISO) to 9.36 Log PFU/mL, reaching a level comparable to that of the CM. Similarly, pEa_47 showed an increase from 8.27 Log PFU/mL (IO) under optimized conditions to 9.63 Log PFU/mL after the addition of supplements (ISO), closely matching the control (CM) yield of 9.67 Log PFU/mL. The yield of Fifi318 improved from 8.39 Log PFU/mL (IO) to 9.88 Log PFU/mL with the addition of supplements (ISO), surpassing

Table 2 Analysis of variance (ANOVA) results for the production of *Erwinia* phages pEa_SNUABM_8, pEa_SNUABM_27, and pEa_SNUABM_31

	DF	Adj sum of squares	Adj mean square	F value	*P* value
pEa_SNUABM_8					
Regression	5	360,505	72,101	13.41	0.000
A	1	132,110	132,110	24.57	0.000
B	1	194,845	194,845	36.24	0.000
A^2	1	7690	7690	1.43	0.241
B^2	1	4171	4171	0.78	0.385
AB	1	19,980	19,980	3.72	0.063
Residual error	31	166,688	5377		
Lack of fit	19	128,267	6751	2.11	0.094
Pure error	12	1,238,421	3202		
Total	38	530,649			
pEa_SNUABM_27					
Regression	5	53,310	10,662	5.76	0.001
A	1	17,650	17,650	9.53	0.004
B	1	15,592	15,592	8.42	0.007
A^2	1	1163	1163	0.63	0.434
B^2	1	11,276	11,276	6.09	0.019
AB	1	8357	8357	4.52	0.041
Residual error	31	57,398	1852		
Lack of fit	19	31,113	1638	0.75	0.724
Pure error	12	26,285	2190		
Total	38	119,375			
pEa_SNUABM_31					
Regression	5	5146	2573	0.21	0.811
A	1	245,106	245,106	20.05	0.000
B	1	26,909	26,909	2.20	0.148
A^2	1	30,576	30,576	2.50	0.124
B^2	1	91,533	91,533	7.49	0.010
AB	1	92,911	92,911	7.60	0.010
Residual error	31	378,956	12,224		
Lack of fit	19	366,313	19,280	16.30	0.057
Pure error	12	12,642	1054		
Total	38	859,210			

the control (CM) yield of 8.62 Log PFU/mL. The most significant improvement was observed for Fifi451, where the yield increased from 7.78 Log PFU/mL in the control (CM) to 9.13 Log PFU/mL (ISO). These results demonstrate that the combined optimization strategy effectively enhanced phage production across different phages, achieving yields comparable to, or exceeding those of the conventional production method.

Optimization of phage production at the fermenter scale

The optimized phage production process, confirmed at the flask scale, was subsequently applied at the fermenter scale as a basis for mass production. Bacterial cultures were set at a concentration of 2×10^5 CFU/mL, and the phages were inoculated at a concentration of 2×10^3 PFU/mL. Additional conditions for application at the fermenter scale were tested using the model phage, pEa_27. The effect of pH adjustment, maintaining levels of 5, 6, 7, and 8, was compared to a control with an unadjusted pH. After 18 h of culture, the group maintained at pH 6 showed a significant 38% increase in production yield. However, the phage production yield decreased by 55%, 37%, and 35% at pH 5, 7, and 8, respectively, compared to that of the control (Fig. 5a). The addition of dissolved oxygen decreased phage production by 26%, although this difference was not statistically significant, indicating a downregulatory effect (Fig. 5b). Based on these results, maintaining the pH at 6 was established as an additional condition for optimal phage production.

Validation of mass-production conditions at the fermenter scale

The model phage, pEa_27, along with pEa_47, Fifi318, and Fifi451 were used to validate the feasibility of mass production at the fermenter scale. The bacterial concentration was uniformly set at 2×10^5 CFU/mL, and that of the phage was set at 2×10^3 PFU/mL, with the selected supplements of fructose and sucrose applied to the fermenter. Further optimization for mass production involved comparing a control group with an unadjusted pH to a group maintained at pH 6. All groups showed a significant increase in phage production compared to that of the control. Specifically, the addition of the pH 6 condition to the optimized settings increased phage production yields increased by 16% for pEa_27, 30% for pEa_47, 303% for Fifi318, and 19% for Fifi451; this allowed the establishment of conditions applicable to fermenter-scale production.

Discussion

The integration of phage therapy within the One Health framework presents a sustainable alternative to agricultural practices, reducing reliance on antibiotics and promoting overall ecosystem health. Traditional control methods for fire blight caused by *E. amylovora* depend heavily on antibiotics, such as streptomycin, oxytetracycline, and oxolinic acid, which are also crucial in veterinary and human medicine [26–28]. The extensive use of these antibiotics in agriculture poses significant environmental risks and can contribute to the development of antibiotic-resistant pathogenic strains. Phage therapy offers a viable alternative to mitigate these risks and promotes a sustainable approach to disease management.

Several studies have characterized newly isolated phages that can effectively infect *E. amylovora* and demonstrate biocontrol effects [21, 29, 30]. Studies that validated the potential of phage cocktails for fire blight control have recently been reported in East Asia (South Korea) [21, 31] and Europe (Spain). Commercialized products, such as Agrihage-Fireblight (USA), containing

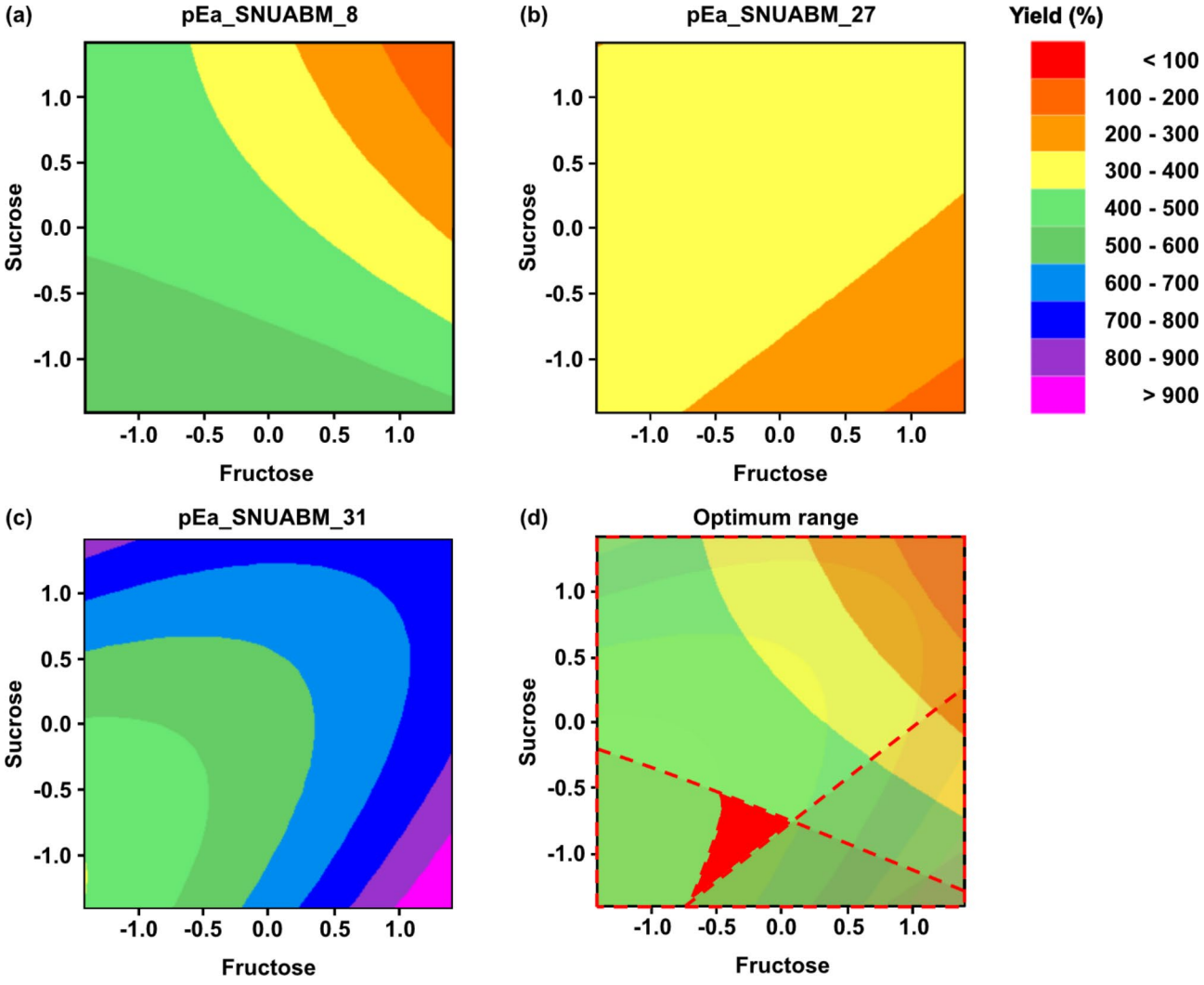

Fig. 3 Response surface 2D contour plots showing the effects of sugars on *Erwinia amylovora* phage production. The effect of fructose and sucrose on the production of pEa_SNUABM_8 (**a**), pEa_SNUABM_27 (**b**), and pEa_SNUABM_31 (**c**) phages were assessed. (**d**) Overlapping coded ranges of media supplements optimized for the three model phages using response surface methodology

10^{12} PFU/L mixed phage strains, exemplify the application of phage therapy in controlling fire blight outbreaks. The company recommends spraying phages at a 200× dilution per acre (~4050 m^2) weekly. This requires 4×10^{17} PFUs weekly in cases where public control for fire blight prevention are implemented, such as in South Korea, which is an enormous quantity [32]. Considering the yield of 10^9–10^{10} PFU/mL for *E. amylovora* bacteriophages, similar toother phages [33], producing the required 100 tons per week presents significant challenges. Typically, phage propagation protocols involve culturing bacterial cells in the early exponential phase (~10^8 CFU/mL) and inoculating them with phages at an MOI ranging from 0.01 to 0.1 [33, 34]. Scaling this process to a working volume of 1 ton requires approximately 10^{14} bacterial cells and 10^{12}–10^{13} phage particles for inoculation. Achieving phage yields above 10^{10} PFU/mL is challenging for many phages, resulting in wasted phages and causing a strain on time and budgetary resources. Consequently, seed culture fermentation often precedes the main culture to generate sufficient inoculums of bacterial hosts and phages [35]. In the case of South Korea, where public control is regulated, 10^{17} CFU bacteria would be needed, which would increase the spread rate of pathogens causing legal communicable diseases such as fire blight. When working with plant-lethal pathogens, it is crucial to minimize pathogen use to reduce leakage risks and optimize inoculum levels to streamline seed culture processes.

To address these challenges, extensive research has been conducted on phage production at the laboratory scale [18, 36]. Effective phage production requires a thorough understanding of bacterial growth and phage infection under optimal conditions, including temperature,

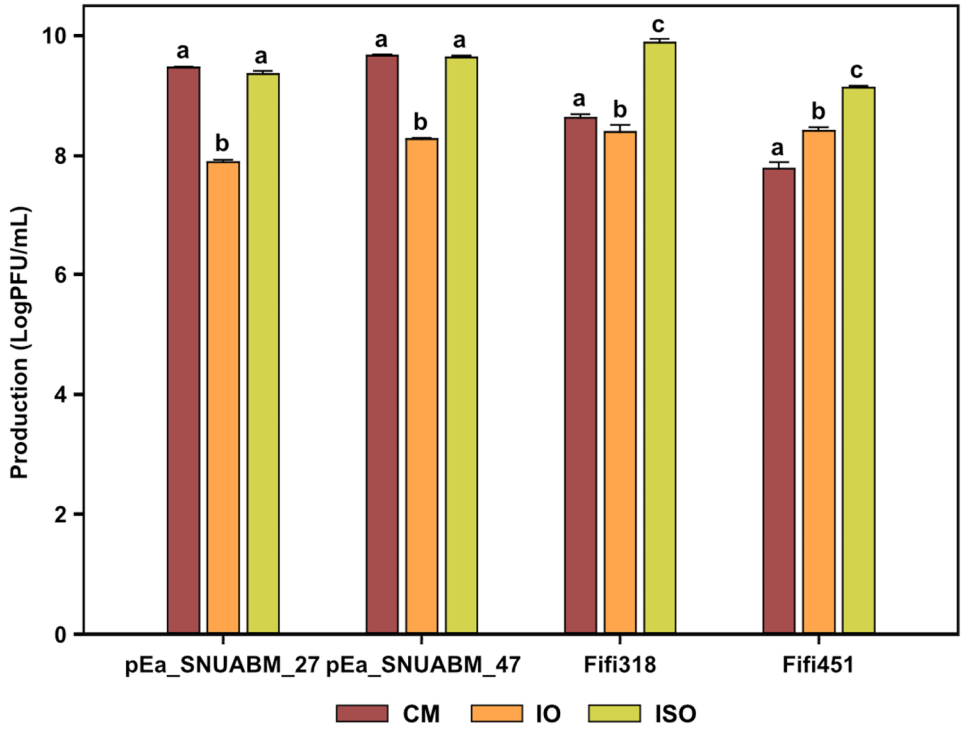

Fig. 4 Validation of optimized phage production conditions at the flask scale using additional *Erwinia amylovora* phages. Yields for the additional *E. amylovora* phages, pEa_SNUABM_27, pEa_SNUABM_47, Fifi318, and Fifi451, were compared under conventional methods, optimized inoculum concentrations (IO), and optimized conditions (ISO), including both phage inoculation and media supplementation. Statistical analysis was performed using the Holm–Sidak test, and statistically significant differences between groups are indicated by different letters above the bars ($p < 0.05$)

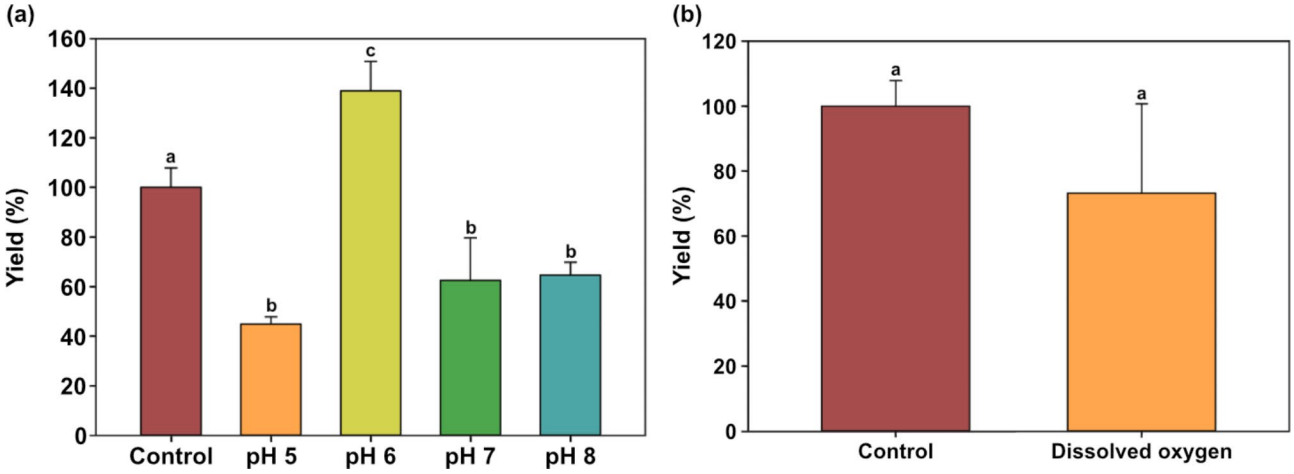

Fig. 5 Further optimization of phage production conditions at the fermenter scale. The pH (**a**) and dissolved oxygen levels (**b**) were assessed. Significance testing was conducted using the Holm–Sidak test, with different letters above the bars indicating statistically significant differences between groups ($p < 0.05$)

MOI, and supplements such as carbon, nitrogen, and ion sources, along with surfactants [14, 37]. We aimed to identify the optimal inoculum levels required for producing commercially viable phages by reducing bacterial concentrations used in the fermentation broth to 10^5 CFU/mL. Our optimization began with small-scale experiments focusing on carbon sources and phage inoculum concentrations. We tested three model phages (pEa_8, pEa_27, and pEa_31) and found that lower phage inoculum levels significantly increased the phage yield (Fig. 1). For example, pEa_31 showed remarkable yield increases of 587.31% at 4 Log PFU/mL and 1,206.99% at 3 Log PFU/mL compared to those of the control. The highest production yield was observed at an inoculum concentration of 3 Log PFU/mL (MOI 0.01), indicating that phage amplification was more efficient at lower initial

inoculum concentrations. This result aligns with findings by Agboluaje and Sauvageau [38], who reported optimal phage production at low initial MOIs (0.01–0.0001) with a bacterial inoculum of 7 Log CFU/mL. Conversely, extremely low MOIs (e.g., <0.00001), have been reported to result in suboptimal phage production [14, 39, 40]. As observed by Kim et al., at low MOIs, each bacterial cell is expected to undergo multiple rounds of infection before lysis, promoting exponential phage amplification [14]. In contrast, extremely low MOIs can limit initial infection events, reducing overall yield due to a slower amplification rate. At high MOIs, multiple phages infect each cell, leading to rapid depletion of bacterial hosts and limiting subsequent replication cycles.

Media supplements have long been used to optimize phage production, with carbon and nitrogen sources, cations, and surfactants being the most common choices. These supplements create favorable conditions for bacterial growth and enhance phage adsorption to host cells, resulting in increased phage propagation [41]. For instance, fructose has been shown to improve the yield of *Limosilactobacillus reuteri* phages by 150% [42]; sucrose increased *E. amylovora* phage production by up to 500% [43]; glucose enhanced *Escherichia coli* phage yield by 60% [44]; and glycerol boosted *Staphylococcus aureus* phage production by 100% [14]. Generally, a positive correlation between bacterial growth and phage propagation was observed, suggesting that the improved growth of *E. amylovora* would likely benefit phage production in our model. Fructose, glucose, and sucrose, which are abundant sugars in apple plants, have been shown to enhance the growth rate of *E. amylovora* strain TS3128 [45]. Notably, all optimizing conditions for phage production (i.e. media supplements, pH, or temperature) showed no detrimental effect on the infectivity of any of the model phages (Fig. S1). Sucrose and fructose led to an overall increase in phage production in the current study and were prioritized for optimization using RSM. In contrast, divalent cations produced inconsistent results (Fig. 2) in our phage pool (Fifi318, Fifi451, pEa_27, and pEa _47), although numerous studies have confirmed their role in enhancing phage propagation [14, 44, 46]. Generally, divalent cations such as Ca^{2+} are considered to increase phage adsorption [46, 47]. For instance, with phage MR-10, Ca^{2+} was associated with increased adsorption and burst size, while also inhibiting host growth [47]. However, as discussed above, premature host cell death can lead to a lower final phage yield.

To scale up production, further optimization of parameters, such as agitation, temperature, and aeration, is necessary. Optimization of factor ranges for phage mass production was achieved using RSM, as depicted in the 2D contour plot (Fig. 3). González-Menéndez et al. used RSM to optimize factors, such as phage and bacterial inoculum, temperature, and agitation, to produce phages in nonpathogenic strains, resulting in an inoculum of 9.3 LogPFU/mL [15]. Grieco et al. applied RSM to optimize fermentation parameters, including temperature, dissolved oxygen, and pH [16]. Kim et al. used RSM to enhance media supplements and achieved significant increases in yield [14]. Our study confirmed the versatility of RSM for phage production optimization, with fructose and sucrose as key supplements showing the potential for application in other *Erwinia* phages to boost yields (Figs. 3 and 4).

One of the key advantages of using a bioreactor is its adjustability, enabling the host cell cycle to be maintained under ideal conditions for phage production. For example, bacteriophage T4 can be continuously produced in *E. coli* (ATCC 11303) by synchronizing the host cells at an optimal stage, achieving a yield of 1.45×10^{10} PFU/mL [35]. Other studies have refined T4 production by modifying phage growth parameters, such as the latent period and burst size, resulting in yields of 10^9 PFU/mL/h (2.4×10^{13} PFU/day) [48]. In this study, further optimization was achieved using a fermenter system by adjusting the pH and dissolved oxygen levels to improve the growth conditions of host bacteria as discussed above. Phage production peaked at a pH of 6, which was the most favorable condition for *E. amylovora* (Fig. 5) [49, 50]. As observed by Grieco et al., additional oxygenation did not increase the yield further, ultimately reaching a 6% yield compared to the non-oxygenated condition [51]. The optimized conditions for the fermenter were validated across our phage pool (Fifi318, Fifi451, pEa_27, and pEa_47), demonstrating the versatility of the model, which achieved yields within the commercializable range of 10^9–10^{10} PFU/mL (Fig. 6).

Despite efforts to mitigate safety concerns, such as the evolution of resistant mutants during scale-up, large-scale production still relies on host cells, which introduces several challenges. These challenges encompass both endogenous and exogenous factors that must comply with good manufacturing practices, including the purification of toxins and host metabolites (e.g., exo-/endotoxins), the presence of prophages and phage contaminants, risks associated with using pathogenic host cells, and regulatory considerations regarding the use of legal pathogens (host cells) in phage replication [19, 52]. To address these limitations in scaling up, in vitro synthesis of phages is expected to offer a promising alternative to bypass the issues arising from the use of cells that may possess pathogenic features [10, 53, 54]. The optimization of phage production is vital for its industrial application, especially in agriculture and aquaculture, where phage solutions are used in large amounts for environmental rather than individual treatments. In this study, we aimed to develop a cost-effective phage production

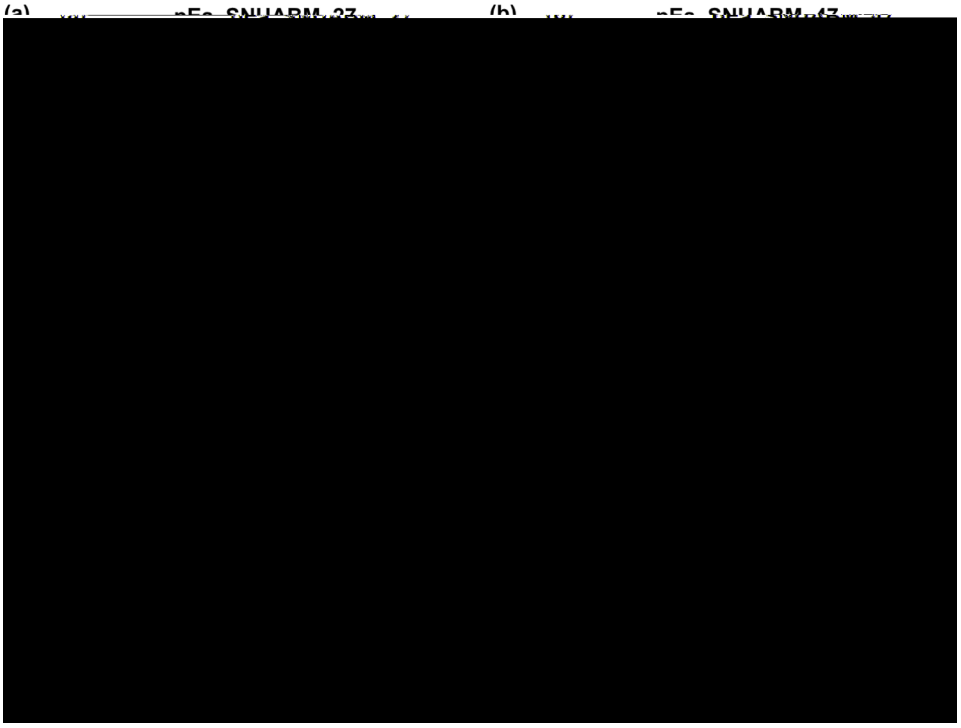

Fig. 6 Validation of large-scale fermenter production conditions using various *Erwinia amylovora* phages. The *E. amylovora* phages, pEa_SNUABM_27 (**a**), pEa_SNUABM_47 (**b**), Fifi318 (**c**), and Fifi451 (**d**), were tested. The optimized phage production conditions were applied with additional parameters to maximize yields at the fermenter scale. Statistical significance was evaluated using the Holm–Sidak test, with different letters above the bars indicating significant differences between groups ($p < 0.05$)

strategy through inoculum optimization, thus reducing the spread of hazardous bacteria and the need for extensive seed phage and bacterial fermentation.

Conclusions

In conclusion, this study demonstrates the potential of optimizing phage production for agricultural applications, especially in controlling fire blight caused by *E. amylovora*. By significantly reducing the inoculum size (1,000-fold for bacteria and 10,000-fold for phages), employing RSM to optimize media supplements, and using advanced fermenter technology, we achieved phage production yields comparable to those of conventional methods. Our findings demonstrate that phage production can be efficiently scaled to commercializable concentrations (10^9–10^{10} PFU/mL) under optimized conditions, with peak yields observed at optimal pH levels for host cell growth. The versatility of the model was validated across different phages in our pool (Fifi318, Fifi451, pEa_SNUABM_27, and pEa_SNUABM_47), suggesting its broad applicability. However, further research is required to refine large-scale phage production, particularly to ensure biosafety when handling plant-lethal pathogens, such as *E. amylovora*. Overall, this study lays the groundwork for developing cost-effective and scalable phage production strategies that could contribute to sustainable agricultural practices and reduce the reliance on antibiotics.

Abbreviations

ANOVA	Analysis of variance
CCD	Central composite design
CFU	Colony-forming units
CM	Conventional method
IO	Phage inoculum optimization
ISO	Phage inoculum and supplement optimization
MOI	Multiplicity of infection
NB	Nutrient Broth
PFU	Plaque-forming units
RSM	Response surface methodology

Supplementary Information

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Supplementary Material 1

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Not applicable.

Author contributions

SGK conceptualized the idea and designed the project. SJJ, SSG, SBL, and YML performed the experiments. SJJ, YML, WJJ, JHP, MHH, DP, EJP, SWK, JWJ, and SGK performed data analysis. SJJ, and ER interpreted the data. SCP and SGK wrote the manuscript. All the authors reviewed the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations
Not applicable.

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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