J Ind Microbiol Biotechnol (2012) 39:1515–1522
DOI 10.1007/s10295-012-1148-3

Optimization of fermentation parameters in phage production using response surface methodology

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Received: 24 January 2012 / Accepted: 4 May 2012 / Published online: 20 June 2012

Abstract Previously, we used computer-controlled fermentation technology to improve the yield of filamentous phage produced in *Escherichia coli* by 10-fold (Grieco et al., Bioprocess Biosyst Eng 32:773–779, 2009). In the current study, three major fermentation parameters (temperature, dissolved oxygen [DO], and pH) were investigated using design of experiments (DOE) methodology. Response surface methodology (RSM) was employed to create a process model and determine the optimal conditions for maximal phage production. The experimental data fitted best to a quadratic model ($p < 0.0001$). Temperature and pH, but not DO, proved to be significant variables. The model predicted a theoretical optimal condition for maximal bacteriophage production at temperature of 28.1 °C and pH 6.9. A validation run resulted in phage production [3.49×10^{11} transducing units (TU)/mL] comparable to the predicted value (2.86×10^{11} TU/mL). This represented a 7-fold increase in phage production above that obtained without optimization, resulting in a 70-fold increase above that achieved by shake flask culture alone.

Keywords Design of experiments · Response surface methodology · Central composite design · Fermentation process optimization · Filamentous phage production

Abbreviations

ANOVA	Analysis of variance
CCD	Central composite design
CI	Confidence interval
CV (%)	Coefficient of variance
DO	Dissolved oxygen
DOE	Design of experiments
R^2	Coefficient of determination
RSM	Response surface methodology

Introduction

Information about key process variables in fermentation such as temperature, pH, and dissolved oxygen (DO) often represents proprietary knowledge and tends not to be published. As a result, in many cases, reproducibility of a fermentation process requires extensive downtime and empirical trials when different locations, time, or individuals are involved. With the advent of the biotechnology industry, there is an increasing demand for researchers to have access to detailed fermentation profiles to provide systematic information to find optimal fermentation conditions for the same or similar systems. However, searching

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for the ideal combination of the fermentation parameters is rarely performed due to time limits and the cost of the fermentation runs. However, the development of the design of experiments (DOE) concept makes such optimization more easily accomplished.

DOE is a statistical analysis of processes to test which variables influence an outcome. A process is defined as a combination of variables such as operations, equipment, methods, individuals, etc. that transform inputs into an output [13]. Recently, DOE methodologies have become a popular tool to optimize bioprocesses such as fermentation runs [11]. The response surface methodology (RSM) is a collection of statistical DOE techniques that are useful for modeling and analysis of processes in which a response of interest is influenced by several variables and the object is to optimize the responses [1, 4, 13, 14, 17]. In most RSM experiments, the relationship between the response and the independent factors is unknown. Central composite design (CCD) is the most popular class of design used for fitting the data to a second-order model. We demonstrated previously that the filamentous bacteriophage yield in a computer-controlled fermenter could be improved by changing important determinants such as pH, DO, and the composition of the culture medium [7]. In this study, the ranges of key fermentation variables (temperature, pH, and DO) were investigated systematically using DOE technology to find functional relations between the independent variables and the theoretical optimal conditions.

Materials and methods

Bacteriophage and bacterial strain

E. coli strain K91 was the host strain used in these studies and is described in Smith and Scott [18]. The bacteriophage "landscape" phage library was constructed and kindly provided by Dr. Valery Petrenko of Auburn University [16]. We previously screened this random peptide P8 library against chalcopyrite ($CuFeS_2$) and identified a peptide sequence of interest [6]. The phage displaying the sequence DSQKTNPS was used in all of the fermentation experiments in the current study.

Media and inoculum

NZY medium was used for all fermentations as it was previously shown to be the optimal medium for phage production (when compared with Luria Broth or Super Broth) [7]. NZY broth was prepared as follows: 10 g N-Z-Amine-A (Sigma-Aldrich, Oakville, Ontario, Canada), 5 g yeast extract (BD Biosciences, Mississauga, Ontario, Canada), and 5 g NaCl in 1 L distilled H_2O, and the pH adjusted to 7.4. NZY plates contained NZY broth with 11 g/L select agar (Invitrogen, Burlington, Ontario, Canada) and 20 μg/mL tetracycline (Sigma-Aldrich). Inocula and media for all DOE optimization experiments (20 experiments) were prepared at the same time to minimize variation between fermentations. NZY broth (18 L) was prepared as above but without pH adjustment and stored at 4 °C during optimization experiments. Medium (700 mL) was poured into each bioreactor and autoclaved, and the pH was adjusted to individual set points immediately before inoculation. No contamination was observed in any of the media during the experiments.

A bacteriophage-infected colony of *E. coli* strain K91 was selected from an agar plate and added to 300 mL NZY broth containing 20 μg/mL tetracycline. The culture was shaken at 250 rpm and 30 °C overnight. The overnight culture was mixed with 60 mL 50 % glycerol, dispensed into 10-mL aliquots in 15-mL Falcon tubes, and stored at −80 °C. An inoculum of 10 mL was thawed and used for subsequent fermentation runs.

Fermentation runs

Fermentation equipment was located in the UBC Centre for Blood Research Fermentation Suite. Bioreactors, biocontrollers, and pH and DO sensors were purchased from Applikon Biotech Inc. (Foster City, CA). Fermentation was performed as previously described [7] with slight modifications. Three bioreactors were set up at one time; the total number of fermentations for this study was 22; 20 fermentations were performed for DOE optimization, and an additional 2 fermentations were used for validation runs to compare the current and optimal conditions. The "current condition" was defined as temperature of 37 °C, pH of 7.4, and DO of 100 % [7]. All fermentations were performed in 700 mL medium using 3-L Applikon bioreactors. The second six-blade impeller was removed from the motor shaft because the mixing was sufficient with a single impeller. In addition, the location of the upper blade was just above the volume of the medium and would have caused excessive foaming of the medium [5]. pH probes were calibrated before sterilization with two standard buffers, pH 4.0 and pH 7.0. pH was controlled by using a proportional–integral–derivative (PID) control system with the following parameters (as recommended by the manufacturer): P-gain of 50, integral and derivative time of zero, dead zone of 0.05, bias of 0, and cycle time of 30. Temperature and pH were adjusted to set points, and the partial pressure of oxygen in the medium was calibrated to 100 % by purging the medium with filtered air at flow rate of 1 L/min for 30 min. Agitation for all fermentations was fixed at 400 rpm. The DO level was adjusted to predetermined set points using a solenoid oxygen valve which

provided additional oxygen at flow rate of 2.8 L/min if necessary. No nitrogen was used as a suppressor of DO. The inoculum was taken from −80 °C and allowed to thaw at room temperature for 90 min before addition to bioreactors, and no antifoaming agent was added. Samples were harvested after 15 h of fermentation, at which point cell density and bacteriophage yield were measured.

DOE process optimization

For identification and statistical analysis of the process factors, the software package Design Expert® version 8.0.5 (Stat-Ease Inc., Minneapolis, MN) was used in this study. The three independent factors and their five levels in a full factorial CCD are given in Table 1. The program generated a set of 20 experiments which consisted of eight factorial points, six axial points, and six repeats of the center point. RSM experiments consist of four steps: (1) evaluation of design, (2) experiments, (3) analysis of responses, and (4) optimization [1]. Our design was evaluated to ensure the rotatability of the design space with $\alpha = 1.682$ and six repeats of the center point calculated by the software. Thus, the regions of operability (coded values: $\pm\alpha$) for each factor are wider than those of interests (coded values: ± 1) and had to be considered in advance. No blocking was applied in this study. The experiments were conducted in random order as suggested by the software. The analysis of the experiments was subdivided into five categories: fit summary, analysis of variance (ANOVA), diagnostics, model reduction, and model transformation. The final equation and model graphs were created based on the ANOVA. Criteria for optimization were set to maximize phage production (TU/mL) and were tested by validation runs. This analysis allowed comparison of the predicted and actual values at the theoretical optimal condition, as well as direct comparison of production before and after DOE optimization.

Titration of bacteriophage

High-density ($OD_{595} = 1.0$ to 1.5), noninfected K91 cells (for use in phage titer determination) were freshly prepared each day as previously described [7]. Samples were collected at the end of fermentation (15 h) and then centrifuged at 10,000 rpm for 5 min at room temperature to remove bacteria. The supernatant (containing bacteriophage) was transferred to a new tube, and sodium azide was added to final concentration of 0.02 % (w/v) to prevent further bacterial growth. Dilutions of the bacteriophage were made in NZY medium, and 10 μL of each of the diluted bacteriophage samples was added to 30 μL freshly prepared high-density K91 cells. Samples were incubated for 10 min at room temperature to allow the phage to infect the bacteria. Tetracycline resistance genes were induced by addition of 160 μL NZY medium containing 0.2 μg/mL tetracycline. Samples were then incubated at 37 °C for 30 min with shaking at 200 rpm. Spot titers were performed by adding 30 μL of the phage-infected bacteria to an NZY agar plate containing 20 μg/mL tetracycline. After the drops were absorbed by the agar, the plates were inverted and incubated overnight at 30 °C. The number of tetracycline-resistant transducing units (TU) was determined, and the phage yield was calculated. For each sample, the average of three determinations was used for the analysis of DOE optimization.

Results

Fit summary for model fitting

The values of the three variables (temperature, DO, and pH) and the experimental (actual) and predicted yields of bacteriophage in the 20 fermentation runs are presented in Table 2. The fermentation runs were performed in random order. The actual or predicted data before taking the log transformation are also listed in Table 2. The second-order polynomial model was used to correlate the independent variables with bacteriophage production. The best candidate to fit the data among a linear, two-factor interaction, quadratic, or cubic model was chosen by the fit summary comparison.

As shown in Table 3, the quadratic model was chosen as the best fit model by using a type I sum of squares (sequential sum of squares) analysis which compares the ratio of the mean square regression to the mean square

Table 1 The three factors and their values in a full factorial central composite design space

Factor	Fermentation parameters	−α (−1.682)	Low (−1)	Mean (0)	High (+1)	+α (1.682)
		Actual values				
A	Temperature (°C)	14.2	20.0	28.5	37.0	42.8
B	DO (%)	20.0	40.0	70.0	100.0	120.0[a]
C	pH	2.3	4.0	6.5	9.0	10.7

[a] The DO probe was calibrated to 100 % by air saturation. To increase DO level above 100 %, O_2 was supplemented into bioreactor when necessary

Table 2 Actual and predicted values of phage production

Standard order	Run order	A Temp (°C)	B DO (%)	C pH	Phage (TU/mL)		Ln [Phage (TU/mL)]	
					Actual	Predicted	Actual	Predicted
1	6	20.0	40	4.0	5.20×10^{6}	2.48×10^{10}	15.47	16.61
2	16	37.0	40	4.0	7.45×10^{6}	2.46×10^{10}	15.82	16.18
3	4	20.0	100	4.0	6.37×10^{6}	1.77×10^{12}	15.67	16.61
4	3	37.0	100	4.0	3.72×10^{6}	1.77×10^{12}	15.13	16.18
5	12	20.0	40	9.0	9.48×10^{8}	2.51×10^{10}	20.67	19.96
6	14	37.0	40	9.0	2.32×10^{8}	2.49×10^{10}	19.26	19.53
7	5	20.0	100	9.0	1.11×10^{9}	1.77×10^{12}	20.83	19.96
8	9	37.0	100	9.0	5.73×10^{7}	1.77×10^{12}	17.86	19.53
9	18	14.2	70	6.5	2.28×10^{8}	1.27×10^{12}	19.24	19.41
10	8	42.8	70	6.5	5.93×10^{8}	1.27×10^{12}	20.20	18.68
11	11	28.5	20	6.5	2.97×10^{12}	3.78×10^{12}	28.72	26.25
12	20	28.5	120	6.5	1.01×10^{13}	6.71×10^{12}	29.94	26.25
13	13	28.5	70	2.3	9.00×10^{3}	1.27×10^{12}	9.10	7.50
14	2	28.5	70	10.7	3.98×10^{5}	1.27×10^{12}	12.89	13.14
15	1	28.5	70	6.5	8.85×10^{8}	9.62×10^{11}	20.60	26.25
16	10	28.5	70	6.5	3.77×10^{12}	9.62×10^{11}	28.96	26.25
17	19	28.5	70	6.5	3.35×10^{10}	9.62×10^{11}	24.23	26.25
18	15	28.5	70	6.5	6.24×10^{10}	9.62×10^{11}	24.86	26.25
19	7	28.5	70	6.5	1.28×10^{12}	9.62×10^{11}	27.88	26.25
20	17	28.5	70	6.5	1.81×10^{11}	9.62×10^{11}	25.92	26.25

residuals. The coefficient of variance (CV) is the error expressed as a percentage of the mean. It is computed as 100 × (standard deviation)/(mean). A low CV value (11.19 %) indicates a very high degree of precision and good reliability of the experimental values. The fit of the model can be expressed by the coefficient of determination, R^2, adjusted R^2, as well as predicted R^2. The adjusted R^2 value is a measure of the amount of variation around the mean explained by the model, adjusted for the number of parameters in the model. The adjusted R^2 increases only if additional parameters improve the model more than would be expected by chance [1]. Both the R^2 value (0.88) and the adjusted R^2 value (0.84) were high in this model, indicating that a high percentage of the variability in the response could be explained by the model. The predicted R^2 value was 0.80, indicating the amount of variation in the predicted values that is explained by the model. Adequate precision can be measured using the signal-to-noise ratio, with a ratio >4 being desirable. Here, the ratio of 16.22 indicates an adequate signal.

Diagnostics, model reduction/transformation, and ANOVA

Diagnostics allow the detection of outliers and subsequent transformations of the model if necessary. No outlier was detected by externally studentized residuals (outlier-*t* value, data not shown). The Box–Cox plot suggested a log transformation (lambda = 0), and the model was transformed to a natural log (Ln) scale. Subsequently, the backward model reduction was applied to eliminate the insignificant parameters, and a final equation was determined accordingly.

A quadratic model with three factors can have up to nine significant variances (A, B, C, AB, AC, BC, A^2, B^2, and C^2). Each variance is evaluated by ANOVA, which is based on type III sum of squares (partial sum of squares; see Table 4). The type III sum of squares is the change to the residual sum of squares caused by deleting the individual variances from the full model. The *F*-value was used to compare significance of the model and its variances. A model *F*-value of 26.26 implied that the model was significant, and its *p* value (<0.0001) indicated that there is only a 0.01 % chance that this large model *F*-value could occur due to noise. The same *F*-value was used to find significant variances. If a variable is significant, its variances (terms involving the variable) can have large *F*-values and low *p* values. By allowing backward model reduction, the insignificant variances are eliminated based on their *F*-values and *p* values and only the significant variances are retained in the ANOVA table (Table 4). These are variance C (pH, *p* value of 0.0171), A^2 (quadratic terms of temperature, *p* value of 0.0008), and C^2 (quadratic terms of pH,

Table 3 Model fitting values

Model terms	Values
CV (%)[a]	11.19
R^2 [b]	0.88
Adjusted R^2 [c]	0.84
Predicted R^2 [d]	0.80
Adequate precision[e]	16.22
Standard deviation	2.31

[a] CV (%): coefficient of variance, the standard deviation expressed as a percentage of the mean. It is calculated by dividing the standard deviation by the mean and multiplying by 100

[b] R^2: a measure of the amount of variation around the mean explained by the model. $R^2 = 1 - [SS_{residual}/(SS_{residual} + SS_{model})$

[c] Adjusted R^2: a measure of the amount of variation around the mean explained by the model, adjusted for the number of terms in the model. The adjusted R^2 decreases as the number of terms in the model increases if those additional terms do not add value to the model. Adj $R^2 = 1 - [\{SS_{residual}/df_{residual}\}/\{(SS_{residual} + SS_{model})/(df_{residual} + df_{model})\}]$

[d] Predicted R^2: a measure of the amount of variation in new data explained by the model. Pred $R^2 = 1 - [PRESS/(SS_{residual} + SS_{model})$. The predicted R^2 and the adjusted R^2 should be within 0.20 of each other

[e] Adequate precision: this is a signal-to-noise ratio that compares the range of the predicted value at the design points to the average prediction error. Adequate prediction $= p\sigma^2/n$ (Anderson and Whitcomb [1]). SS: sum of squares, df: degrees of freedom, PRESS: predicted residual sum of square, p: number of model parameters including intercept and any block coefficient; σ^2: residual mean square from ANOVA table; n: number of experiments

Table 4 ANOVA of the model

Source	Sum of squares	Degrees of freedom	Mean square	F value	p value prob. > F
Model	561.71	4	140.43	26.26	<0.0001
A-Temperature	0.63	1	0.63	0.12	0.7352
C-pH	38.45	1	38.45	7.19	0.0171
A^2	94.46	1	94.46	17.66	0.0008
C^2	461.63	1	461.63	86.32	<0.0001
Residual	80.21	15	5.35		
Lack of fit	36.44	10	3.64	0.42	0.8883
Pure error	43.77	5	8.75		
Cor total	641.93	19			

p value of <0.0001). The reason why variance A (temperature) remained in the ANOVA table is that a hierarchy autocorrection for the significant A^2 recovered the variance A. This means some insignificant variances remained in the final equation due to the significance of the variances at the higher hierarchy. However, the variable DO was proven insignificant to the model, and all of its variances (B, AB, BC, or B^2) were removed from the ANOVA table.

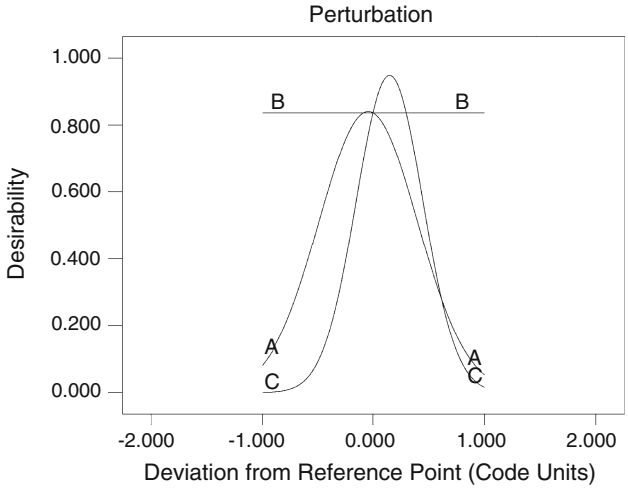

Fig. 1 Perturbation plot, *A*–temperature; *B*–DO; *C*–pH

The center point in our experiments was repeated six times to calculate the pure error in the model (Table 4). A lack-of-fit test compares the residual error to the pure error from replicated design points. A residual error significantly larger than the pure error indicates that something in the residuals could be removed by a more appropriate model. F-value of the lack of fit is calculated as (mean square lack of fit)/(mean square pure error). In this case, the low F-value of lack of fit and the high p value indicated that the lack-of-fit test is insignificant, thereby confirming a good model fit (0.42 and 0.89, respectively).

The perturbation plot (Fig. 1) compared the effect of all the factors at a particular point in the design space. It is equivalent to a one factor at a time (OFAT) experiment; as a result, the plot does not show the effects of interactions between factors. The response was plotted by changing only one factor over its range while holding all the other factors constant. A perturbation plot at the center point (28.5 °C, 70 % DO, and pH 6.5) was obtained to show the relative effect of fermentation parameters on phage production (Fig. 1). The x-axis shows a deviation of the factor values from the reference point (center point) as a coded value. The y-axis shows the desirability of the response (maximum phage value would have a desirability of 1.000). A steep slope or curvature in a factor shows that the response is sensitive to that factor. A relatively flat line indicates insensitivity to change in that particular factor. The perturbation plot indicated that pH (C) is the most influential factor, followed by temperature (A). DO (B) was insignificant, showing insensitivity to the response.

Final equation and model graph

After the regression analysis, coefficients, standard errors, and low/high 95 % confidence intervals (CI) were

Table 5 Coefficient table

Factor	Coefficient estimate	Standard error	95 % CI low	95 % CI high
Intercept	26.25	0.80	24.54	27.96
A-Temperature	−0.22	0.63	−1.55	1.12
C-pH	1.68	0.63	0.34	3.01
A^2	−2.55	0.61	−3.84	−1.26
C^2	−5.63	0.61	−6.92	−4.34

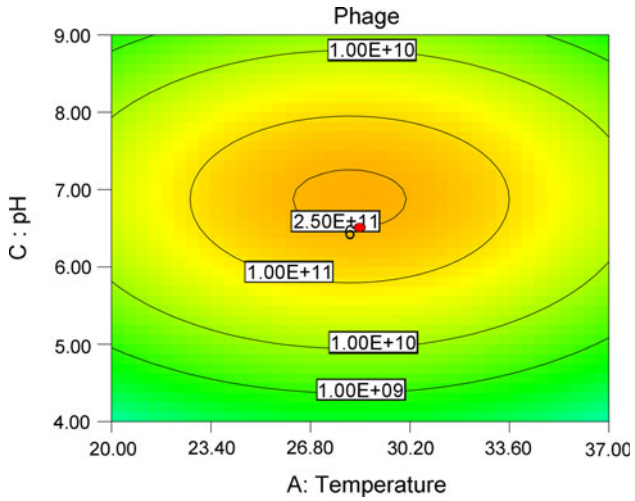

Fig. 2 Contour plot for phage production, *A*–temperature, *C*–pH. The plot shows a center point (*red circle*) with the number of repeats indicated. Phage production: low (*green*) to high (*orange*)

calculated (Table 5). This coded equation can be converted to actual values as follows:

$$\begin{aligned}\mathrm{Ln(Phage)} = {} & -44.10183 + 1.98449 \times \mathrm{Temperature}(^{\circ}\mathrm{C}) \\ & + 12.38517 \times \mathrm{pH} \\ & - 0.035261\ \mathrm{Temperature}\ (^{\circ}\mathrm{C})^2 - 0.90108 \\ & \times \mathrm{pH}^2 \end{aligned} \quad (1)$$

Predicted values of 20 conditions (Table 2) were produced based on this equation. Using the final equation with actual factor levels (1), a predicted value for any condition (in the region of interest) can be calculated. A contour plot for bacteriophage production (Fig. 2) shows the predicted values of the response. The center point is indicated as a red spot, and the number 6 indicates the number of repeats. The maximum phage production (orange) was predicted around the center points of temperature and pH with phage production value $>2.5 \times 10^{11}$ TU/mL.

Optimization and validation

Optimization criteria were set to produce a maximum yield of phage in the regions of interest in each significant factor. The maximum yield of phage was expected under fermentation conditions of 28.1 °C and pH 6.9 with a predicted value of 2.86×10^{11} TU/mL. The three-dimensional map of the response with pH and temperature shows the theoretical optimal condition as well as the current fermentation condition (Fig. 3). Two fermentations were performed as validation runs to compare phage production: one using the theoretical optimal condition (28.1 °C, 70 % DO, and pH 6.9) and the other using the current condition (37.0 °C, 100 % DO, and pH 7.4). To perform fermentations using the optimal condition, the DO for the center point was set at 70 %; although this variable is insignificant, a value in the region of interest (40–100 %) must be used for the response to fit the model. Even when a factor is deemed nonsignificant, a setting must still be chosen. A value in the mid-region of the range could be considered "robust" because variation from the mid-point would not have a detrimental effect on phage production. The actual values were obtained and validated for 90 % prediction and confidence intervals in the "Point Prediction and Confirmation" nodes in the "Optimization" tool. Actual values from the current and optimal conditions were within the intervals (data not shown). This meant that the predicted and the actual values at the optimal condition (2.86×10^{11} TU/mL versus 3.49×10^{11} TU/mL) were comparable. However, the predicted value at the current condition was somewhat lower than the actual value (1.40×10^{10} TU/mL versus 4.95×10^{10} TU/mL). This difference is probably related to the location of the current condition in the design space, being further from the center point than the optimal condition. We designed our model

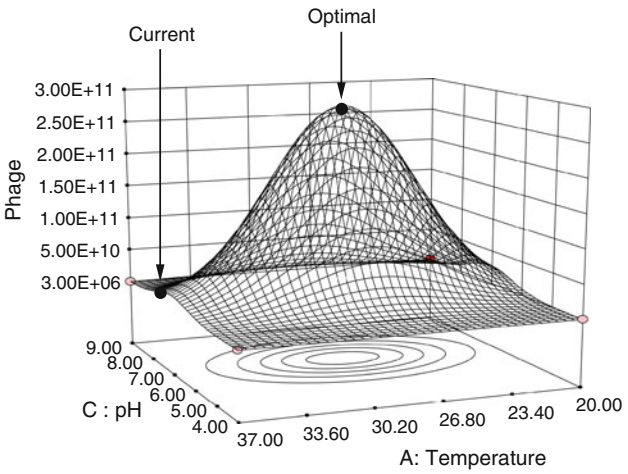

Fig. 3 Comparison of predicted values from the model with optimal fermentation conditions. The model shown is at 100 % DO to indicate the four factorial design points. *Pink*: design points with actual values lower than the predicted values. *Red*: design point with actual values higher than the predicted value. In addition to the four factorial design points, the predicted values of the optimal and current fermentation conditions are indicated in *black*

as "rotatable," which means it has the same predictability between design points equidistant from the center of the design. The closer a design point is to the center of the plot, the better the prediction. The actual value at the current fermentation condition was slightly lower than the previously obtained value (1×10^{11} TU/mL) [7]. This difference is likely due to differences in the length of the fermentation, the speed of agitation, and the different preparation method for the starter cultures. Direct comparison of the two fermentations using the current and optimal conditions showed a 7-fold increase in phage production (4.95×10^{10} TU/mL versus 3.49×10^{11} TU/mL).

Discussion

Media selection and use of computer-controlled fermentation improved production of bacteriophage by up to ten times compared with shake flask culture [7]. In this study, the three major fermentation parameters (temperature, dissolved oxygen level, and pH) were studied for their effects on phage production. The optimized conditions produced 3.49×10^{11} TU/mL of phage, which was comparable to the predicted value of 2.86×10^{11} TU/mL.

pH was the most significant variable for phage production. Based on our model, phage production decreased by about 20 % on changing 0.5 units of pH (data not shown). In contrast, DO was not a significant variable and was removed from the model (p value of 0.9734, data not shown). It is important to note that all fermentations started at 100 % DO (see "Materials and methods" section for details) and no nitrogen was added. Therefore, the only difference between fermentations was the time taken to reach the set point before oxygen was purged into the bioreactors. The DO level at the end of fermentation was above the set point due to nutrient depletion and the absence of N_2 gas. This is a common observation at the end of fermentation when bacterial growth has reached the stationary phase. Our goal was to ensure sufficient oxygen during fermentation rather than maintain the DO set points. Interactions between variables (AB, AC, or BC in the model variances) were shown to be insignificant, indicating that each fermentation parameter affects phage production independently; no synergistic effect between the variables was apparent.

There is very little information published on optimization of key process variables for phage production, although there are some studies on lytic phages and host cell survival at different temperatures and pH [3, 8, 9]. In earlier studies of filamentous phage such as M13, temperature of 32–34 °C was used for growth of host cells while 42–44 °C was used for phage infection (permissive temperature) [10, 12, 15]. Commonly used protocols for filamentous phages currently use 37 °C for both the growth of the host cells and the infection and duplication of phage [2]. It is not clear in our study why a much lower temperature (28.1 °C) produced the highest yield of phage. However, a recent report showed that the maximal amount of phage was produced at 31 °C, yet the maximal display of the foreign fluorescent peptides occurred at 28 °C [19]. These authors emphasized the importance of a balance between the optimal temperature for bacterial growth and the optimal temperature for the translocation pathway for functional protein maturation and phage particle assembly.

In conclusion, the optimal fermentation conditions are process specific even in similar target and host systems and need to be adjusted depending on the experimental goal. The current DOE optimization using three key process variables created a second-order quadratic model and revealed that pH was the most significant variable whereas DO was the least significant variable. The validation runs comparing the optimal and the current conditions in parallel showed an additional 7-fold increase in phage production. This significantly lowers the cost and time commitment involved in phage production and purification.

Acknowledgments The authors thank the UBC Centre for Blood Research Fermentation Suite for providing fermentation facilities. The authors also thank Ms. Shari Kraber (Stat-Ease Inc.) for her advice on the analysis of the DOE data and the preparation of the manuscript. This work was supported in part by grants from the Canadian Institutes of Health Research (to R.T.A.M.) and the National Sciences and Engineering Council (to W.S.D.).

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