Original Research Article

OPTIMIZATION OF *ESCHERICHIA COLI* BACTERIOPHAGES PRODUCTION

ABSTRACT

Aims: Phage therapy may be the best alternative to antibiotics in poultry. There is a need for optimization of phage production conditions to produce more *Escherichia coli* specific phages for poultry applications.

Methodology: An *E. coli* λ bacteriophage isolated in our laboratory was used for optimization studies. An optimization of the *E. coli* λ bacteriophage production process was carried out by conventional methods to identify the factors, and the Thaguchi process was used for the optimization of conditions for increased bacteriophage production. With four factors each at three levels L9, experimental runs were carried out.

Results: In the conventional method, 8 factors were studied and 4 selected for statistical optimization by Taguchi. At optimized conditions of modified nutrient broth with 1% glycerol, temperature 37^{o}C , 100 rpm for 12 hours of phage multiplication, an improvement of the final titer was achieved from a control $2x10^{9}$ to optimized $5x10^{12}$ Plauque formation Units (PFU)/mL.

Conclusion: Phage production is dependent on bacterial growth and bacteriophage multiplication in bacteria. In such a complicated system, the Thaguchi method will be a convenient method of phage production optimization.

Key Words: Bacteriophage, *E. coli*, Phage Therapy, λ Phage, Taguchi

Introduction:

Increased demand for poultry products has forced overuse of antibiotics and is causing resistant microbial infections. Interest has grown in phage therapy as an alternative treatment. Utilization of bacteriophage to kill resistant bacteria is a bright option for poultry to control diseases. Many bacterial phages are reported to control pathogenic *E. coli* and *Salmonella* in poultry. Although the clinical significance of bacteriophages [1] is well established, there are very few clinical trials of phage therapy in commercial poultry [2]. Ecolicide PX™ commercial preparation is only *specific to Escherichia coli O157:H7* [3]. "The efficacy of bacteriophages depends on their good adaptation to replication and survival under the required conditions. To achieve their optimal efficacy, it is advisable to improve methods of phage selection, their isolation from the host environment, and their production in large quantities" [4]. "It should be noted that most phages can be destroyed when exposed to the low pH of the stomach" [5]. "The importance of the phage concentration applied has also been shown in the studies on phage therapy in chickens (>10^{10} PFU/mL) and on poultry products (10^{7} PFU/cm^{2}). The data demonstrate that only high concentrations of phages are effective in ensuring a significant reduction in mortality and in foodborne pathogens" [6]. " In the chickens intracranially infected with *E. coli*, application of a higher dose of the phage, at a titre of 10^{8} PFU, fully protected the birds against the development

of infection" [7]. "Optimization of conditions is a routine process done to maximize productivity" [8]. "Traditionally, optimization involves changing one reaction parameter at a time, while keeping the others at fixed level, known as the one-factor-at-a-time (OFAT) experimental approach" [9]. "However, the major limitation of OFAT is that it fails to consider any possible interaction between factors of each reaction parameter" [10]. "But it will identify the key factors of the process. A comprehensive and reliable optimization method is the design of experiments (DOE) approach. DOE is a statistical technique used to study multiple variables simultaneously" [11]. "The advantage of the DOE as compared to the OFAT method is its ability to predict the interactions of factors" [12]. "Some of the most popular DOE approaches are the Taguchi optimization method, factorial design, and response surface methodology (RSM). Among them, the Taguchi optimization method offers distinct advantages in that many factors can be examined simultaneously using the fewest number of experimental runs possible" [13]. "The Taguchi optimization method has been applied by numerous researchers to optimize the reaction conditions in order to achieve a certain product's quality" [14-16]. Hence, the objective of this study is to maximize the *E. coli* bacteriophage production by optimizing the reaction conditions based on the Taguchi optimization method.

Materials and Methods

Bacterium, Phages and Medium

The present study was conducted with the bacterium *Escherichia coli* isolated from poultry samples [17]. Cultures to be used as inocula were grown overnight in modified nutrient broth at 37°C and 150 rpm in an orbital incubator-shaker to obtain a cell density of approximately 2×10^9 cfu/ml. *E. coli* bacteriophage, isolated in our laboratory [17] was used for production optimization studies.

A modified nutrient broth medium was used for all fermentations for phage production.

Modified nutrient broth was prepared with 10 g peptone, 5g yeast extract, and 5 g NaCl in l L distilled water, and the pH adjusted to 7.0. Modified Nutrient Plates contained Modified Nutrient Broth with 18 g/L Agar Agar.

All microbial media components and chemicals were purchased from Himedia, India.

Viable cell density and phage titer measurements

Viable cell counts were performed on the samples. The samples were diluted in series, and 100-µL dilutions were spread on modified nutrient agar plates. Plates were incubated overnight at 37°C and colonies were counted. The viable cell density was reported as colony forming units per ml (cfu/ml).

Measurements of free phage concentration were performed using the double layer method [18]. Filtered samples (0.2µm syringe-filter) were diluted, and 100µL filtrates were mixed with 100µL L of actively growing bacteria (OD600 0.6), incubated at 37°C for 10 minutes. To the mixture, 5ml soft agar was added and overlayered on modified nutrient agar plates. Plates were incubated

overnight at 37°C. Plaques were counted, and the phage titer was reported as plaque forming units per ml (PFU/ml). The measured number of plaques was converted to PFU (plaque forming units)/mL using the following equation:

PFU/mL = Dilution Factor × Nplaque × Vsample

N plaque represents the number of plaques and V sample represents the volume of the sample.

Phage production at flask level:

Each 200 mL of modified nutrient broth pH 7 was taken in a 500 mL flask and inoculated with 5 mL of pure *E. coli* culture of 0.8 OD600 having 10^8 cells/ml and incubated for 18 hrs. Then 4 ml of λ bacteriophages were added to the flask and incubated at 37^{oC} with a shaking rate of 100 rpm for 18 hours, followed by static for 18 hours for lysis of cells. Then the unlysed cells are removed by centrifugation at 5000 rpm for 10 minutes, and the phage lysate is filtered through a 0.2-micron membrane filter and subjected to a plaque assay. 10,100 microlitres of 10^2 dilution of filtered phage lysate and 100 microlitre of pure culture were mixed , incubated at 37°C for 10 min and mixed with 5 ml of low melting agar medium (0.8%) and poured onto agar plates and incubated at 37°C for 24hrs.

Identification of important factors effect on phage productivity:

To select the important factors affecting phage productivity, we adapted the one-factor-at-a-time method in the beginning. This approach is conducted by altering one nutritional/chemical/physical factor of fermentation, while keeping all the other factors constant. Eight of the factors were selected based on the literature [19-21]. An attempt is made to identify the impact temperature for increased production of phages using a shake flask. Individual batch fermentations were carried out at 25, 30, 37 and 40°C.

In the experiments for testing of the carbon source, modified nutrient broth medium was supplemented with 1% (w/v) of glucose, sucrose, glycerol, and galactose, individually.

To assess the effect of nitrogen sources, modified nutrient broth was supplemented with 1% (w/w) of casamino acid, peptone, tryptone, and glycine, individually. The influence of divalent cations on bacteriophage production was investigated by supplementing 1% of calcium chloride, zinc chloride, manganese chloride, or magnesium chloride to the modified nutrient broth medium.

pH was evaluated by carrying out the fermentation at pHs 5, 6, 7, and 8. Shaking conditions were studied at 0, 100, 150, and 200 rpms in an orbital shaking incubator (Scigenics, Chennai, India), and incubation time was studied by phage multiplication at various time intervals of 6, 12, 18, 24 hrs. After fermentation, phages were assayed by the agar double layer method.

K_2HPO_4 at different concentrations (0, 0.5, 1, and 1.5%) was tested to detect possible buffering conditions on phage production.

Phage production in a 2L flask:

Modified nutrient broth (1.2L) was taken in two 2Lflask and inoculated with 25 ml of pure exponential culture of *E. coli* and incubated at 37^{o}C for 18hrs.The pure phage suspension (10 ml) was added to the flask and incubated at 37^{o}C for 18 hours with a shaking rate of 100 rpm followed by 18 hours of static. Phage lysate is made free of cells by centrifuging at 5000 rpm for 10 minutes, and then filtered through a 0.2 micron syringe filter and undergone a plaque assay.

Phage production: statistical optimization by Thaguchi methodology:

Experimental Design. DOE was applied by adopting the Taguchi statistical design approach. All optimization studies were carried out using a 2L flask with a working volume of 1200 mL in triplicates. This method uses a set of orthogonal arrays, in which reaction parameter optimization is performed using the fewest number of experimental runs possible. The orthogonal array employed had orthogonal arrays with four parameters at three-levels, L-9. The experimental design was built using Taguchi statistical software (Qualiteck 4). The objective criterion or the response chosen for increased bacteriophage production The four reaction parameters identified manually and to be optimized at their respective three levels are presented in Table 1.

Table 1. Design of experiments, with four parameters at three levels for the production parameters optimization L9

	Factors	Units	Level1	Level2	Level3
1	Temperature	^{o}C	25	30	37
2	Shaking	RPM	050	100	150
3	Glycerol	%	0.5	1	1.5
4	Incubation time	hr	4	8	12

The diversity of factors was studied by crossing the orthogonal array of the control parameters, as shown in Table 2.

Table 2. L9 Inner array

Experiment No/Factor	1	2	3	4
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	2	1	2	3
5	2	2	3	1
6	2	3	1	2
7	3	1	3	2
8	3	2	1	3
9	3	3	2	1
Total	18	18	18	18

"Larger is better" is chosen as the goal is to maximize the phage's production. Results obtained from the L-9 experimental runs were fed to Qualiteck 4 software, which further analyzed the analysis of variance (ANOVA) using the signal-to-noise (S/N) ratio. Lastly, the optimum combination of operating conditions predicted by the Taguchi method was tested for validity by running a confirmation reaction at the optimum predicted operating conditions.

Confirmation of Trial-Page Production

For a confirmation production trial, modified nutrient broth with 1% glycerol was taken in two 2L flasks with a working volume of 1.2 L and inoculated with 20 ml of a pure exponential culture of *E. coli*. Flasks were incubated at 37^{o}C, 100 rpm for 18 hours. Six ml of pure phage suspension was added to the flask and incubated at 37^{o}C for 12 hours with a shaking rate of 100 rpm, followed by 18 hours of static. Phage lysate is made free of cells by centrifuging at 5000 rpm for 10 minutes, and then filtered through a 0.2-micron syringe filter and submitted to a plaque assay.

Results

Shake Flask Experiments for Phage Production

In a 500 ml shake flask experiment with 200 ml of modified nutrient broth at 37^{o}C, with 100 rpm, pH 7, inoculum of 4 ml, and an incubation of 18 hrs 5±0.5X10^{9} PFU for the *E. coli* phage were obtained (Figure 1).

Figure 1: *E. coli* bacteriophage plaques

In 2L shake flask experiment of 1200ml modified nutrient broth at 37^{o}C, with 100rpm, pH 7, *E. coli* inoculum of 25ml and 10 ml phage and incubation of 18 hrs, 2±0.3X10^{9} PFU was produced. A small reduction in productivity was observed from 200 to 1200ml working volume.

Identification of important factors effecting on Phage productivity.

Four levels of 8 factors (pH, Temperature, Incubation time, Carbon source, Nitrogen source, K_2HPO_4, divalent cation and shaking conditions) were studied individually to identify the effectiveg factors on *E. coli* λ bacteriophage production.

From the manual experiments four important factors (Temperature, Agitation, Glycerol content and incubation time) were identified for *E. coli* λ bacteriophage production (Table 3).

Table 3: Selection of factors by one-factor-at-a-time method.

Factor	4 Levels and Phage productionin PFU			
pH	5 $8\pm0.1X10^7$	6 $1\pm0.3X10^9$	7 $5\pm0.2X10^9$	8 $4\pm0.5X10^8$
Temperature	**25 $2\pm0.3X10^4$**	**30 $8\pm0.4X10^7$**	**37 $5\pm0.4X10^9$**	**40 $4\pm0.3X10^7$**
Incubation time	**6 $2\pm0.5X10^6$**	**12 $8\pm0.6X10^{10}$**	**18 $4\pm0.3X10^9$**	**24 $2\pm0.2X10^8$**
Carbon source	**Glucose $4\pm0.4X10^9$**	**Sucrose $3\pm0.2X10^9$**	**Glycerol $4\pm0.4X10^{10}$**	**Galactose $2\pm0.5X10^9$**
Nitrogen source	casamino acid $8\pm0.3X10^9$	Peptone $5\pm0.2X10^9$	Tryptone $7\pm0.4X10^9$	Glycine $5\pm0.4X10^9$
Divalent cation	Calcium $1\pm0.5X10^{11}$	Zinc $2\pm0.2X10^8$	Manganese $8\pm0.3X10^7$	Magnesium $5\pm0.2X10^7$
Shaking	**0 $5\pm0.5X10^7$**	**100 $4\pm0.1X10^9$**	**150 $8\pm0.2X10^9$**	**200 $2\pm0.2X10^{10}$**
K_2HPO_4	0 $5\pm0.3X10^9$	0.5 $1\pm0.2X10^9$	1 $3\pm0.5X10^9$	1.5 $2\pm0.2X10^9$

Thaguchi optimization

The L9 orthogonal array of Taguchi method has been used for DOE optimization process. Four factors of each three levels were chosen (Table 1) and experiments were designed based on inner array provided by Qualiteck-4 software (Table 2).

Influence of Factors

Three readings of each L9 trial was fed to Qualiteck4 and analyzed by S/N ratio (Bigger the best) to get impact of each factor and production performance. It was found that Temperature at 37°C, Shaking at 100rpm, Glycerol content at 1% and incubation time of 12hrs were significantly affecting the productivity when compared with other levels (Figures 2&3). Glycerol and incubation time were influencing more for *E. coli* phage production.

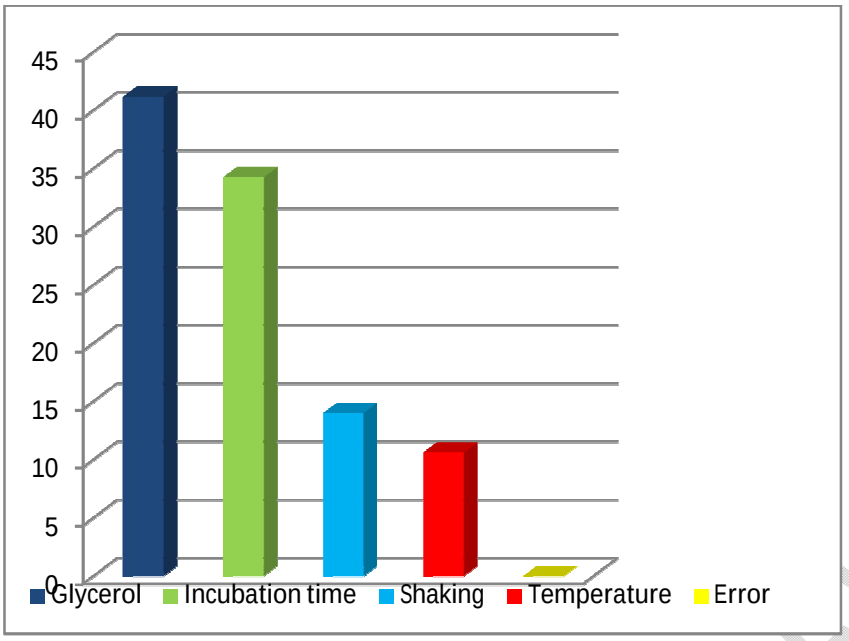

Figure 2. Effect of different factors on *E. coli* phage production

Impact of levels of factors

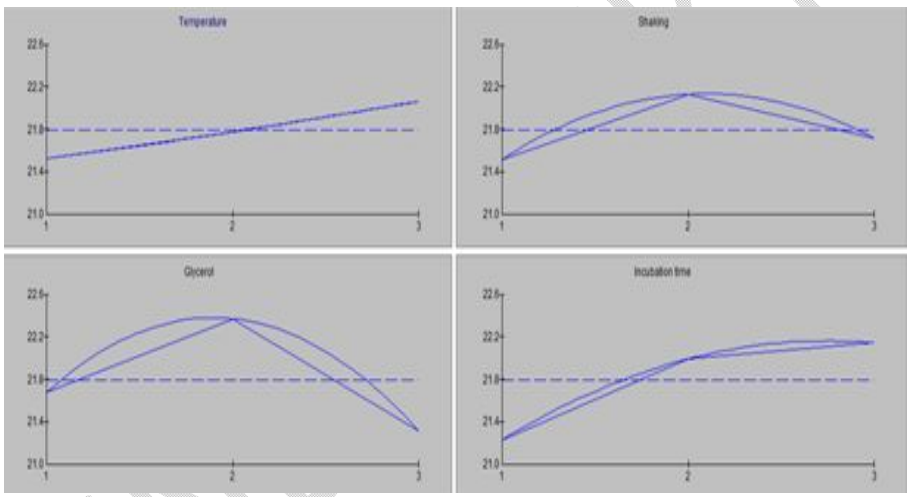

Figure 3: Effect of different levels of factors on *E. coli* Phage production

Interactions of Selected Factors

For *E. coli* phage production optimization Temperature, Shaking and Glycerol interactions were significantly effecting. Hence Temperature was taken as optimization even showing little individual effect (Table 4).

Table4. Number of Interactions between two factors calculated for *E. coli* phage

	Interacting Factor Pairs	columns	SI(%)	Col	Opt
1	Temperature x Shaking	1 x 2	82.76	3	[1,2]
2	Shaking x Glycerol	2 x 3	45.04	1	[2,1]
3	Temperature x Glycerol	1 x 3	34.28	2	[1,2]
4	Temperature x Incubation	1 x 4	32.94	5	[1,2]
5	Shaking x Incubation time	2 x 4	14.85	6	[1,2]
6	Glycerol x Incubation time	3 x 4	14.01	7	[1,3]

Analysis of variance (ANOVA)

Three readings of each L9 trial were fed to Qualiteck4 and analyzed by S/N ratio (Bigger the best) to get ANOVA.

Table 5. ANOVA table of *E. coli* bacteriophage production

	Column /Factor	DOF (f)	Sum of sqr (S)	Variance (V)	F-Ratio (F)	Pure Sum (S^I)	Percent P(%)
1	Temperature	2	.485	.242	-----	.485	10.597
2	Shaking	2	.641	.32	-----	.641	13.985
3	Glycerol	2	1.887	.943	-----	1.887	41.167
4	Incubation time	2	1.569	.784	-----	1.569	34.247
	Other Error	0					
	Total	8	4.583				100.00%

There is zero error and maximum contribution of Glycerol and Incubation time for *E. coli* phage production (Table 5).

Computation of Optimum conditions

Glycerol was found to contribute 60.9%, incubation time 37%, shaking at 100rpm 35.8% and temperature 28.9% on productivity in *E. coli* bacteriophage (Table 6).

Table6. Contribution of factors and increased productivity for *E. coli* bacteriophages production

	Column /Factor	Level Description	Level	Contribution
1	Temperature	37	3	.289
2	Shaking	100	2	.358
3	Glycerol	1%	2	.609
4	Incubation time	12hr	3	.37

Total contribution from all factors 1.626

Current grand average of performance 21.794

Expected result at optimum condition 23.42

Phages production minimum 8% production enhancement is expected.

Optimum parameters

From the analysis using Qualiteck4 software, the following optimum parameters were obtained for bacteriophage production: shaking at 100 rpm, Shaking 100rpm, Glycerol 1%, bacteriophage incubation time of 12 hours, and optimum temperature of 37^{o}C.

Confirmation test

At optimized conditions, confirmation trials were conducted for *E. coli* bacteriophage production in triplicates. The bacteriophages produced were $5\pm0.2X10^{12}$ PFU. *T*he values are greater than the control and any of the individual factors used in the study.

Discussion

In poultry, bacterial infections are challenging [22, 23]. Uncontrolled usage of antibiotics in poultry is leading to the development of antibiotic resistant bacteria [24, 25]. Bacteriophages offer great potential as an alternative to antibiotics in poultry [26]. Bacteriophages effectively kill resistant bacteria to reduce the prevalence of antibiotic resistance [27, 28]. *E. coli* and *Salmonella* are the predominant microbial pathogens in commercial poultry [29]. *E. coli* strains are known for high chicken mortality and have been found to be reduced by phages [30, 31]. In a study by Barrow et al. [32], bacteriophage R, was effective in preventing and treating infections in chickens. Huff et al. [33] have demonstrated that an aerosol spray of bacteriophages administered to 7-day-old chickens prior to the triple challenge with *E. coli* can prevent infections caused by *E. coli*. Tawakol et al. [34] showed that bacteriophage treatment reduced the severity and prevented mortality. Samah Eid et al [35] reported the greatest lytic activity of phages against the *E. coli* strains at 10^{7} concentrations in 24 h and reduction of *E. coli* biofilm within 12 h. Grieco et al [36] optimized a phage produced in *Escherichia coli* by 10-fold using computer-controlled fermentation technology.

There is very little information published on the optimization of key process variables for phage production. The rotatable central composite design (RCCD) methodology was used, combining and comparing all parameters to determine the ideal conditions for the production process of bacteriophages (37). Directly influenced by environmental conditions like medium composition, temperature, pH, and agitation [38, 39, 40]. Temperature and pH, but not DO, proved to be significant variables [41]. Hence, we adopted one factor at a time for short listing of factors, and then DOE Taguchi was used to optimize the phage production conditions. Four levels of 8 factors were studied individually to identify the effective factors on *E. coli* λ bacteriophage production. From the manual experiments, four important factors were identified for *E. coli* λ bacteriophage production. These four shortlisted factors were used by Taguchi to optimize the production process. Taguchi DOE is a powerful tool for optimizing the performance characteristics of a process. In the present study, the goal is to evaluate the effects of process parameters on the performance measure and the optimum combination of control factors that would maximize phage production. The selection of control factors and their levels is made on the basis of manual optimization, one factor at a time. Four factors, such as temperature, shaking, glycerol content, and incubation time, were selected for the study. Each of the four factors was treated at three levels. Three levels of each factor have been made because the effect of these

factors on the performance characteristic may vary. An orthogonal array is a fractional factorial design with pairwise balancing properties. An L9 standard orthogonal array is chosen for the present investigation S/N ratio with higher is the better. Glycerol was found to contribute 60.9%, incubation time 37%, shaking at 100 rpm 35.8% and temperature 28.9% on productivity in *E. coli* bacteriophage. Incubation time influenced phage production. With the increment in incubation time resulting in a corresponding increase in phage production, probably due to a characteristic physiological state of the cells growing [40, 42].Agitation is a crucial aspect of phage production. Agitation plays a role in the aeration dynamics and oxygen availability, increases the probability of contact between the phage particles and the host cells, leading to an irreversible attachment and beginning the infection process [43, 44]. However, if the agitation is too intense, the phage tails can be broken while the absorption process inhibits the infectious process [45].Temperature is less important individually but shows good interactions with other factors, so it should be taken in an optimized condition. Temperature effects on bacterial growth also influence phage production (46). At optimized conditions of modified nutrient broth with 1% glycerol, temperature 37^{o}C, 100 rpm for 12 hours of phage multiplication, an improvement of the final titer was achieved from a control $2x10^{9}$ to optimized $5x10^{12}$PFU/mL.Phage production depends on bacterial growth and bacteriophage multiplication in bacteria. In such a complicated system, the Thaguchi method will be convenient method of production optimization.

Conclusion: Phage production is dependent on bacterial growth and bacteriophage multiplication in bacteria. In such a complicated system, the Thaguchi method will be a convenient method of phage production optimization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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