

Original Research Article  
**OPTIMIZATION OF *ESCHERICHIA COLI* BACTERIOPHAGES  
PRODUCTION**

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**ABSTRACT**

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

**Methodology:** *E. coli*  $\lambda$  bacteriophage isolated in our laboratory used for optimization studies. An optimization of the *E. coli*  $\lambda$  bacteriophage production process was carried out by conventional method to identify the factors and Taguchi process was used for optimization of conditions for increased bacteriophage production. With 4 factors each at three levels L9, experimental runs were carried out.

**Results:** In 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°C, 100 rpm for 12hrs of phage growth an improvement of the final titer was achieved from control  $2 \times 10^9$  control to optimized  $5 \times 10^{12}$  PFU/mL.

**Conclusion:** Phage production is depended on bacterial growth and bacteriophage growth in bacteria. In such a complicated system Taguchi method will be convenient method of production optimization.

**Key Words:** Bacteriophage, *E. coli*, Phage Therapy,  $\lambda$  Phage, Taguchi

**Introduction:**

Increased demand of poultry products forced to over usage of antibiotics, and causing resistant microbial infections. Interest has grown for phage therapy as an alternative treatment. Utilization of bacteriophage to kill the resistant bacteria is a bright option for poultry to control the diseases. Many bacterial phages are reported to control pathogenic *E. coli* and *Salmonella* in poultry. Although clinical significance of bacteriophage [1] is established there are very few clinical trials of phage therapy in commercial poultry [2]. Ecolicide PX™ commercial preparation for only specific to *Escherichia coli* O157:H7 [3]. In a study of Barrow et al. [4], bacteriophage R, was effective in preventing and treating infections in chickens. Huff et al. [5] have demonstrated that 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. [6] showed that bacteriophage treatment reduced the severity and prevented the mortality. The efficacy of bacteriophages depends on their good adaptation to replication and survival in required conditions. To achieve their optimal efficacy, it is advisable to improve methods of phage selection and their isolation from the host environment and production in large quantity [7]. It should be noted that most phages can be destroyed when exposed to low pH of the stomach [8]. The importance of the phage concentration applied has been also shown in the studies on phage therapy in chickens ( $>10^{10}$  PFU/mL) and on poultry products ( $10^7$  PFU/cm<sup>2</sup>). The data demonstrate that only high concentrations of phages are effective in ensuring a significant reduction in mortality and in foodborne pathogens [9]. In the chickens intracranially infected

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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 [10]. Optimization of conditions is a routine process done to maximize the productivity [11]. Traditionally, optimization involves changing the one reaction parameter at a time, while keeping the others at fixed level, known as the one-factor-at-a-time (OFAT) experimental approach [12]. However, the major limitation of OFAT is that it fails to consider any possible interaction between factors of each reaction parameter [13]. 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 [14]. The advantage of DOE as compared to the OFAT method is its ability to predict the interactions of factors [15]. Some of the most popular DOE approaches are Taguchi optimization method, factorial design and response surface methodology (RSM). Among them, Taguchi optimization method offers distinct advantages in which many factors can be examined simultaneously using the least number of experimental runs possible [16]. Taguchi optimization method has been applied by numerous researchers to optimize the reaction conditions in order to achieve certain product's quality [17-19]. 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 [20]. Cultures to be used as inocula were grown overnight in modified nutrient broth at 37°C and 150 rpm in an incubator-shaker (orbital) to get the cell density of approximately  $2 \times 10^9$  cfu·mL<sup>-1</sup>. *E. coli* bacteriophage  $\lambda$  bacteriophage isolated in our laboratory [20] was used for production optimization studies.

Modified Nutrient broth medium was used for all fermentations for phage production.

Modified Nutrient broth was prepared with 10 g peptone (Himedia) -, 5g yeast extract (Himedia), and 5 g NaCl in 1 L distilled H<sub>2</sub>O, and the pH adjusted to 7.0. Modified Nutrient plates contained Modified Nutrient broth with 18 g/L agar agar.

### Viable Cell Density and Phage Titer Measurements

Viable cell counts were performed for samples. The samples were diluted in series and 100- $\mu$ L 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<sup>-1</sup>).

Measurements of free phage concentration were performed using double layer method [21]. Filtered samples (0.2 $\mu$ m syringe-filter) were diluted and 100 $\mu$ L filtrates were mixed with 100 $\mu$ L active growing bacteria (OD<sub>600</sub> 0.6), incubated constant at 37°C for 10 minutes. To mixture 5ml soft agar was added and over layered 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 ( $\text{pfu}\cdot\text{ml}^{-1}$ ). The measured number of plaques was converted to PFU (plaque forming units)/mL using the following equation:

$$\text{PFU/mL} = \text{Dilution Factor} \times \text{N plaque} \times \text{V sample}$$

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

#### **Phage production at flask level:**

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

#### **Identification of important factors effect on Phage productivity:**

To select the important factors effecting on phage productivity, 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 all the other factors are kept constant. Eight factors were selected based on the literature [22-24]. An attempt is made to identify the impact temperature for more production of phages using shake flask. Individual batch fermentations were carried out at  $25^{\circ}$ ,  $30^{\circ}$ ,  $37^{\circ}$  and  $40^{\circ}\text{C}$ .

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 and incubation time was studied by phage growth at various time intervals of 6, 12, 18, 24 hrs. After fermentation phages were assayed by agar double layer method.

#### **Phage production in 2l flask:**

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

#### **Phage production Statistical optimization by Thaguchi methodology:**

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**Experimental Design.** DOE was applied by adopting the Taguchi statistical design approach. All optimization studies were carried out using 2L flask working volume of 1200ml in triplicates. This method uses a set of orthogonal arrays, in which reaction parameters optimization is performed using the least number of experimental runs possible. The orthogonal array employed was 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 bacteriophages 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	°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 when the goal is to maximize the phages production. Results obtained from the L-9 experimental runs were **fed** to Qualiteck 4 software, further analyzed analysis of variance (ANOVA) using signal-to-noise (S/N) ratio. Lastly, the optimum combination of operating conditions predicted by Taguchi method was tested its validity by running a confirmation reaction at the optimum predicted operating conditions.

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### Confirmation trial-Phage Production

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

### Results

#### Shake Flask Experiments for phage production

In 500ml shake flask experiment of 200ml modified nutrient broth at 37°C, with 100rpm, pH7, inoculum of 4ml and incubation of 18 hrs  $5 \times 10^9$  PFU for *E. coli* phage was obtained (Figure 1).



Figure 1: *E. coli* bacteriophage plaques

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

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#### Identification of important factors effecting Phage productivity.

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

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

### 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 feeded 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.

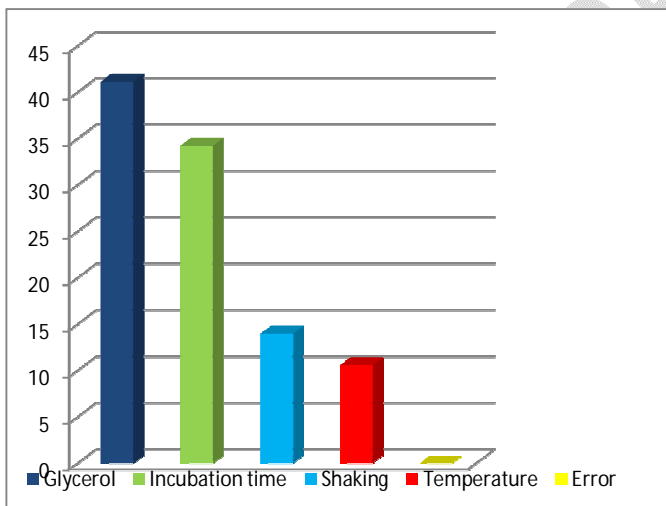


Figure 2. Effect of different factors on *E. coli* phage production

Impact of levels of factors

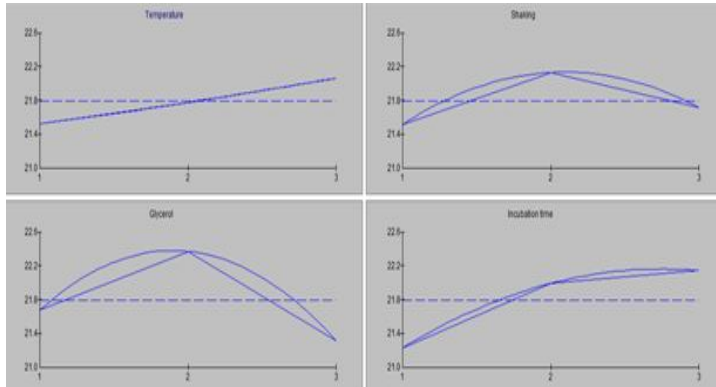


Figure 3: Effect of different levels of factors on *E. coli* Phage production

### 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.

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

	Column /Factor	DOF (f)	Sum of sq (S)	Variance (V)	F-Ratio (F)	Pure Sum (S <sup>1</sup> )	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 Qualiteck4 software the following optimum parameters were obtained for bacteriophages production, Shaking 100rpm, Glycerol 1% and bacteriophage incubation time of 12 hrs and Optimum temperature was 37°C.

### Confirmation test

At optimized conditions confirmations trials were conducted for *E. coli* bacteriophages production in triplicates. Bacteriophages produced were  $5 \times 10^{12}$  PFU. The values are greater than the control and any of individual factors used to study.

### Discussion

In poultry bacterial infections are challenging [25, 26]. Uncontrolled usage of antibiotics in poultry is leading to development of antibiotic resistant bacteria [27, 28]. Bacteriophages offer great potential as an alternative to antibiotics in poultry [29]. Bacteriophages effectively kill resistant bacteria to reduce the prevalence of antibiotic resistance [30, 31]. *E. coli* and *Salmonella* are predominant microbial pathogens of commercial poultry [32]. *E. coli* strains are known for high mortality of chickens and found to be reduced by phages [33, 34]. Samah Eid et al [35] reported the greatest lytic activity of phages against the *E. coli* strains at  $10^7$  concentration in 24 h and reduction of *E. coli* biofilm within 12 h. Grieco et al [36] optimized phage produced in *Escherichia coli* by 10-fold using computer-controlled fermentation technology.

There is very little information published on optimization of key process variables for phage production. Temperature and pH, but not DO, proved to be significant variables [37]. Hence we have 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 (pH, Temperature, Incubation time, Carbon source, Nitrogen source, Calcium divalent cation and shaking conditions) were

studied individually to identify the effective factors on *E. coli*  $\lambda$  bacteriophage production. From the manual experiments four important factors (Temperature, Agitation, Glycerol content and incubation time) were identified for *E. coli*  $\lambda$  bacteriophage production. These four short listed factors were used to optimize the production process by Taguchi. Taguchi DOE is a powerful tool for optimizing the performance characteristic 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 the phages production. Selection of control factors and their levels are made on the basis of manual optimization one factor at a time. Four factors such as Temperature, Shaking, Glycerol content and Incubation time are 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 property. An L9 standard orthogonal array is chosen for the present investigation S/N ratio used is the higher is the better. 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. Temperature is less contributing individually but showing good interactions with other factors hence should be taken in optimized condition.

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At optimized conditions of modified nutrient broth with 1% Glycerol Temperature 37°C, 100 rpm for 12hrs of phage growth an improvement of the final titer was achieved from control  $2 \times 10^9$  control to optimized  $5 \times 10^{12}$  PFU/mL.

Phage production is depended on bacterial growth and bacteriophage growth in bacteria. In such a complicated system Thaguchi method will be convenient method of 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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