OPTIMIZATION OF SALMONELLA BACTERIOPHAGES PRODUCTION BY TAGUCHI APPROCH

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Abstract

Aims: Bacteriophages are a promising alternative to antibiotics in poultry. As more phages are required for phage therapy, ,there is a need for optimization of phage production conditions to produce more *Salmonella enterica* specific phages for poultry applications. **Methodology:** An *S.enterica* SAL-PG bacteriophage isolated in our laboratory was used for optimization studies. The 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 were selected for statistical optimization by Taguchi. At optimised conditions of modified nutrient broth with 1% glycerol, temperature 37^{o}C, 100 rpm for 24 hours of phage multiplication, an improvement of the final titer was achieved from a control $2x10^{9}$ to optimized $8x10^{12}$ Plauque Formation Units (PFU)/mL. **Conclusion:** Phage production is dependent on bacterial growth and bacteriophage multiplication in bacteria. It is proven that in such a complicated system, the Thaguchi method is a convenient method of phage production optimization.

Running title: Optimization of Salmonella Bacteriophages Production

Keywords: Bacteriophage , *Salmonella enterica,* Antibiotic resistance ,Phage therapy ,SAL-PG Phage, Taguchi

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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1. Introduction

Escherichia coli and *Salmonella* sps. infections in poultry are leading to huge losses to poultry and issues of public health [Rahaman et al. 2014]. *Salmonella* infections in poultry may cause pulloram disease (*Salmonella pulloram*), foul typhoid (*Salmonella gallinarum*), foul paratyphoid, etc. [Berchieri et al. 2001]. *Salmonella enterica* serovars, *Enteritidi*s and *Typhimurium*, isolated from poultry products, are the most recurrent causes of acute gastroenteritis [Antunes et al. 2016; Shivaning Karabasanavar et al. 2020]. To control the microbial load in commercial poultry, various antibiotics are being used, which is responsible for the increased emergence of antibiotic-resistant bacteria. Bacteriophages are potential therapeutic agents for bacterial diseases because they have high specificity and lyse target bacteria [Nabil et al. 2018]. Alternative strategies should be attempted to avoid the prevalence of microbial resistance [Golkar et al. 2013; Jun et al. 2014; Fish et al. 2016; Adebayo et al. 2017]. Phage therapy can be a potential alternative to antibiotic treatment and proper control of multi-drug resistant bacteria [Pereira et al. 2016; Park and Nakai 2003; Kutter et al. 2015; Cooper et al. 2016]. Low inherent toxicity, lack of cross-resistance with antibiotics, versatility, high sensitivity, and cell lysis (Nabil 2018) are the advantages of phages in both therapy and food safety [Malik et al. 2017, Principi et al. 2019]. The use of *Salmonella* bacteriophages as biocontrol agents has gained significant interest. A major worldwide problem in combating salmonellosis is the rapidly growing antibiotic resistance [Cohen et al. 2020]. Although the clinical significance of bacteriophages [Dufour et al. 2017] is well established, there are very few clinical trials of phage therapy in commercial poultry [Gorski et al. 2017]. The bacteriophages efficacy depends on their adaptation to environment and replication. For optimal efficacy, it is advisable to select phage from the host environment, and their production in large scale [Garcia et al. 2008]. Generally most phages can be destroyed when exposed to the low pH of the stomach [Zbikowska et al. 2020]. "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). Only higher concentrations of phages are effective in ensuring a significant reduction in mortality of food borne pathogens [Bigot et al. 2011]. Intracranial infection with *Salmonella*, application of a higher dose of the phage, at a titer of 10^{8} PFU, fully protected the chickens [Wernicki et al. 2017]. Optimization of conditions is an important process to maximize productivity[Rabitz et al. 2011]. "Traditionally, optimization involves changing one reaction parameter at a time, while keeping the others at a fixed level, known as the one-factor-at-a-time (OFAT) experimental approach" [Moos et al. 1997]. However, the major limitation of OFAT is that it fails to consider any possible interaction between factors of each reaction parameter [Montgomery 2009]. A comprehensive and reliable optimization method is the design of experiments (DOE) approach. Using DOE multiple variables can be studied simultaneously [Roy 2001]. Using DOE it is possible to predict the interactions of factors [Anderson 1997]. 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 [Houng et al. 2006]. The Taguchi optimization method has been applied by numerous researchers to optimise the reaction conditions in order to achieve a certain product's quality" [Kim et al. 2010;

Kassim et al. 2011; Yaakob et al. 2009**]**. In view of the above, the present study was undertaken to maximise *Salmonella enterica* bacteriophages production by optimizing the reaction conditions based on the Taguchi optimization method.

2 MATERIALS AND METHODS

2.1 Bacterium, Phages and Medium

The present study was conducted with the bacterium *Salmonella enterica* isolated from poultry samples in our laboratory earlier [Hari Narayana Kola et al., 2023a]. Cultures to be used as inoculums 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. *Salmonella enterica* bacteriophage SAL-PG isolated in our laboratory [Hari Narayana Kola et al., 2023a] was used for production optimization studies. A modified nutrient broth was prepared with 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl in 1 L of distilled water, and the pH was adjusted to 7.0. All microbial media components and chemicals were purchased from Himedia, India.

2.2 Viable Cell Density and Phage Titer Measurements

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 count was reported as colony forming units per ml (cfu/ml).

Measurements of free phage concentrations were performed using the double layer method. Filtered samples (0.2µm filtered) were diluted, and 100µL filtrates were mixed with 100µL of actively growing bacteria (OD600 0.6), incubated at 37°C for 10 minutes. To the 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 (PFU/ml). The measured number of plaques was converted to PFU (plaque forming units)/mL using the following equation:

$$PFU/mL = \text{Dilution Factor} \times N_{plaque} \times V_{sample}$$

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

2.3 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 *Salmonella enterica* culture of 0.8 OD600 having 10^8 cells/ml and incubated for 18 hrs. Then 4 ml of SAL-PG bacteriophages were added to the flask and incubated at 37^{o}C 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 and 100 microliters of 10^2 dilutions of filtered phage lysate and 100 microlitre of pure culture were mixed and incubated at 37^{o}C for 10 min, mixed with 5 ml of low melting agar medium (0.8%), poured onto agar plates, and incubated at 37^{o}C for 24 hours.

2.4 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 [Ergin Firuze et al., 2020]. To evaluate the impact of temperature on the increased production of phages using a shake flask, individual batch fermentations were carried out at 25, 30, 37 and 40^{o}C.

In the experiments for testing 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 bacteriophages 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. K_2HPO_4 at different concentrations (0, 0.5, 1, and 1.5%) was tested to detect possible buffering conditions for phage production. After fermentation, phages were assayed by the agar double layer method.

2.5 Phage Production in a 2L Flask

Modified nutrient broth (1.2 L) was taken in two 2L flasks and inoculated with 25 ml of pure exponential culture of *Salmonella enterica* and incubated at 37^{o}C for 18 hours. The pure SAL-PG 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, then filtered through a 0.2 micron syringe filter and subjected to a plaque assay.

2.6 Phage Production: Statistical Optimization by Taguchi Methodology

Experimental design: DOE was applied by adopting the Taguchi statistical design approach. All optimization studies were carried out using 2L flasks 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 (Qualitek 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	^{o}C	25	30	37
2	Shaking	RPM	050	100	150
3	Glycerol	%	0.5	1	1.5
4	Incubation time	hr	6	12	24

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 Qualitek 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.

2.7 Confirmation of Trial-Page Production

For a confirmation production trial, modified nutrient broth with 1% glycerol was taken in three 2L flasks with a working volume of 1.2L and inoculated with 20 ml of a pure exponential culture of *Salmonella enterica*. Flasks were incubated at 37^{o}C, 100 rpm for 18 hours. Six ml of pure SAL-PG phage suspension was added to the flask and incubated at 37^{o}C for 24 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.

2.8 Statistical Analysis

Experiments were repeated three times in triplicates (n=9) and the average values were provided in the results. Standard deviation was calculated with Microsoft excel, ANOVA was calculated with Taguchi Qualitek 4 software.

3 Results

3.1 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 SAL-PG *Salmonella* phage were obtained (Fig. 1).

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

Fig. 1. *Salmonella* **plaques.**

3.2 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 effective factors on *S. enterica* SAL-PG bacteriophages production.

From the manual One factor at a time experiments four important factors (Temperature, Shaking, Glycerol content and incubation time) showing good impact on phage production were identified for *S. enterica* SAL-PG bacteriophages production (Table 3).

Table 3 Selection of factors by one-factor-at-a-time method, Phage production at various conditions.

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

3.3 Taguchi 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 Qualitek-4 software (Table 2).

3.4 Influence of Factors

Three readings of each L9 trial was fed to Qualitek4 and analyzed by S/N ratio (Bigger the best) to get impact of each factor on production performance. It was found that Temperature at 37^{o}C, Shaking at 100rpm, Glycerol content at 1% and incubation time of 24hrs were significantly affecting the productivity when compared with other levels (Fig. 2&3). Glycerol and incubation time were influencing more for *S. enterica SAL-PG* phage production.

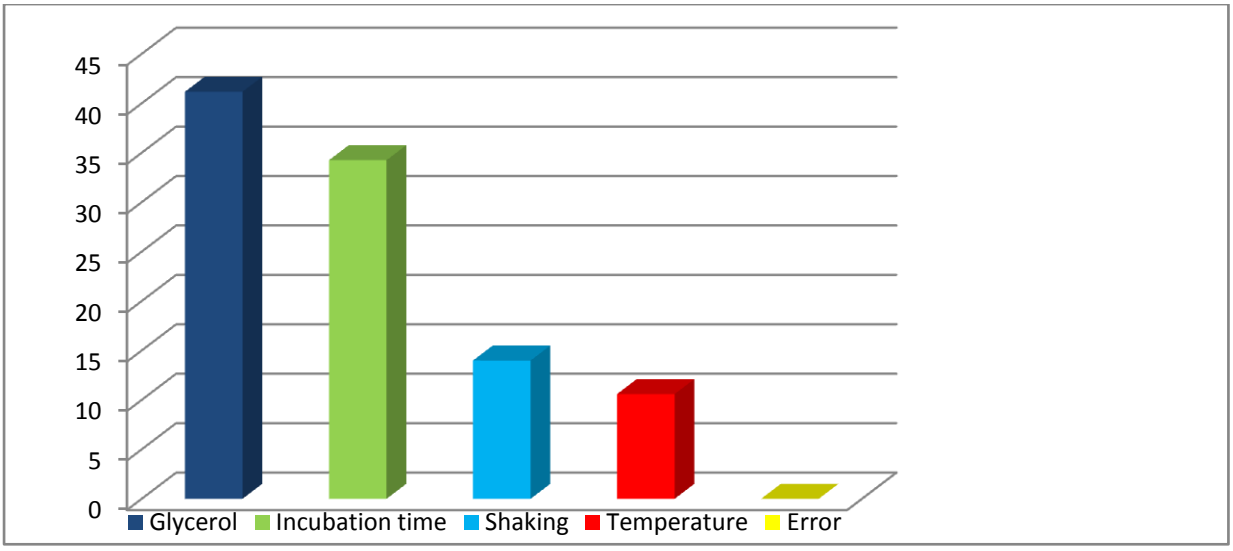

Fig. 2. Effect of different factors on *Salmonella* phage production.

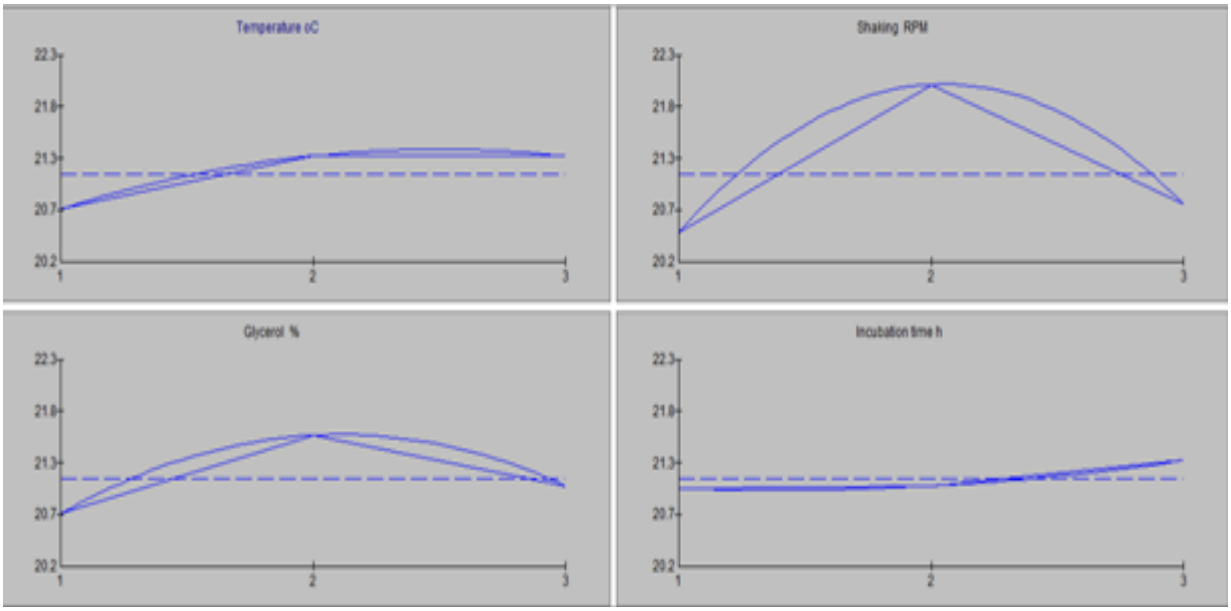

Fig. 3. Effect of different levels of factors on *Salmonella* Phage production.

3.5 Interactions of Selected Factors

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

Table 4 Number of Interactions between two factors calculated for *S. enterica* phage production.

	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]

3.6 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. There is zero error and maximum contribution of Glycerol and Incubation time for *S.enterica SAL-PG* phage production (Table 5).

Table 5 ANOVA table of *S. enterica SAL-PG* bacteriophages 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%

3.7 Computation of Optimum Conditions

By using Qualitech-4 software performances of individual factors was calculated and optimum conditions were derived of it. Glycerol was found to contribute 44.3%, incubation time 18.2%, shaking at 100rpm 92.2% and temperature 18.2% on productivity in *S. enterica SAL-PG* bacteriophages (Table 6). Minimum 8% phages production enhancement is expected.

Table 6 Contribution of factors and increased productivity for *S. enterica* bacteriophages production.

	Column /Factor	Level Description	Level	Contribution
1	Temperature	37	2	.182
2	Shaking	100	2	.922
3	Glycerol	1%	2	.443
4	Incubation time	24hr	3	.182

Total contribution from all factors 1.729

Current grand average of performance 21.112

Expected result at optimum condition 22.814

3.8 Optimum Parameters

From the analysis using Qualitek4 software, the following optimum parameters were obtained for bacteriophage production: shaking at 100 rpm, Glycerol 1%, bacteriophage incubation time of 24 hours, and optimum temperature of 37^{o}C.

3.9 Confirmation Test

At optimized conditions, confirmation trials were conducted for *S. enterica* bacteriophages production in triplicates. The SAL-PG bacteriophages produced were $8 \pm 0.3X10^{12}$ PFU. The values are greater than the control and any of the individual factors used in the study.

4. Discussion

In poultry, bacterial infections are challenging [Galie et al. 2018, Moye et al. 2018]. Uncontrolled usage of antibiotics in poultry is leading to the development of antibiotic resistant bacteria [Allocati et al. 2013, Iredell et al. 2016]. Bacteriophages may offer great potential as an alternative to antibiotics in poultry [Gill and Hyman 2010]. Bacteriophages effectively kill resistant bacteria to reduce the prevalence of antibiotic resistance [Rivas et al. 2010, Sharma et al. 2009]. *E. coli* and *Salmonella* are the predominant microbial pathogens in commercial poultry [Havelaar et al. 2010]. In a study by Barrow et al. [1998], bacteriophage R, was effective in preventing and treating infections in chickens. Tawakol et al. [2019] showed that bacteriophages treatment reduced the severity and prevented mortality. Grieco et al. [2009] optimized and enhanced the phage production in *Escherichia coli* by 10-fold using computer-controlled fermentation technology. There is sparse information on the optimization of key process variables for phage production. In our earlier studies, optimization of E.coli λ phage was carried out using the Thaguchi approach (Hari Narayana Kola et al. 2023b). Silva et al. [2021] used rotatable central composite design (RCCD) methodology for optimizing conditions for the production process of bacteriophages. Environmental conditions like medium composition, temperature, pH, and agitation were studied [Grieco et al. 2012; Padfield et al. 2020]. Temperature and pH, but not DO, proved to be significant variables [Sung-Hye et al. 2012]. Hence, we adopted one factor at a time for the short listing of factors, and then DOE Taguchi was used to optimise the phage production conditions. Four levels of 8 factors were studied individually to identify the effective factors on *S. enterica* SAL-PG bacteriophages production. From the manual experiments, four important factors were identified for *S. enterica* SAL-PG bacteriophages production. These four shortlisted factors were used by Taguchi to optimise 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 as earlier process (Hari Narayana Kola et al. 2023b). Each of the four factors was treated at three levels. Three levels of each factor have been established because the effect of these factors on the performance characteristic may vary. An orthogonal array is a fractional factorial design with pair wise 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 *S. enterica* bacteriophages. 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 [Zaburlin et al. 2017; Abedon et al. 2001]. In E. coli phage production, an incubation time of 12 hrs was optimum (Hari Narayana Kola et al. 2023b) whereas for Salmonella phage it is 24 hrs, may be due to the smaller size of the SAL-PG phage than E .coli λ phage (Hari Narayana Kola et al. 2023a). 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 [Ward et al. 2019; Wittmann and Becker 2013]. 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 [Doyle and Schoeni 1984]. Glycerol was found to contribute 60.9% of productivity in *E. coli* phage whereas in *S. enteric* phage it is 44.3% Only. Shaking at 100 rpm contributed 92.2% of productivity in *S. enteric*, whereas in *E. coli* it is 35.8% (Hari Narayana Kola et al. 2023b). At optimised conditions of modified nutrient broth with 1% glycerol, at 37^{o}C, 100 rpm for 24 hours of phage multiplication, an improvement of phage titer was achieved from a control $2x10^{9}$ to an optimized $8x10^{12}$PFU/mL similar to an earlier study with EColi phage optimization (Hari Narayana Kola et al. 2023b).

DECLARATION OF COMPETING INTEREST: Authors have declared that no competing interests exist.

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