clean cells
SOLUTION FOR BIOLOGICS

UPSTREAM PROCESS OPTIMIZATION OF BACTERIOPHAGE PRODUCTION USING DESIGN OF EXPERIMENT

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ABSTRACT

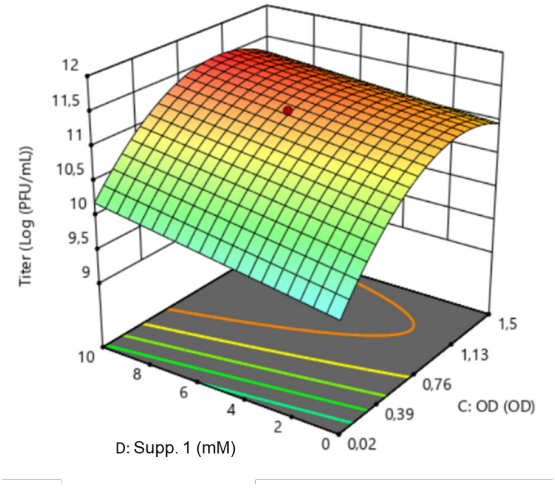
To address multi-resistant bacterial infections, interest has regrown for phagotherapy as an alternative or complementary treatment. While compassionate use of bacteriophages already showed cases of remission (N. Dufour et al, 2017), clinical trials are still at their early stages (Górski et al, 2020).

To encourage clinical trials and ensure exploitable data, it is essential that the final product's quality, efficacy, and safety are guaranteed. Having resort to risk-based approaches such as Quality by Design (QbD) is much encouraged by the Food and Drug Administration and other regulatory authorities (Yu LX et al., 2014). One of the objectives of the QbD is to define a design space in which the product quality is guaranteed. This design space can be achieved with a Design of Experiment (DoE) based optimization, which helps identifying the optimal conditions as well as critical process parameters.

In this purpose, the process development department of Clean Cells has worked on implementing a robust and flexible DoE for upstream process development (USP). The following main objectives were to be achieved:

- To reach the highest phage titers within a reduced time frame
- To gain knowledge on robustness and repeatability
- To be able to select the most interesting condition depending on any specific constraints (technical, cost, robustness, time)
- To be applicable to any other phage

In this work, an optimization of the phage production process was carried out on T7 and T4 bacteriophages, both specific to *Escherichia coli.* The capabilities of the models to predict the phage titers were proven to be effective, which indicates that this approach can be used for further projects and applications. An improvement of the final titer was achieved on both phages, with an increase from 9.5 to 11.2 log_{10}PFU/mL for T7 and 7.9 to 11.2 log_{10}PFU/mL for T4. A design space for each phage was defined in which the phage production appeared to be robust and the phage quality guaranteed. With this data, the scale-up strategy could be secured as well as the manufacturing process.



INTRODUCTION & OBJECTIVES

To address multi-resistant bacterial infections, interest has regrown for phagotherapy as an alternative or complementary treatment. While compassionate use of bacteriophages already showed cases of remission (N. Dufour et al., 2017), clinical trials are still at their early stages (Górski et al, 2020).

To encourage clinical trials and ensure exploitable data, it is essential that the final product's quality, efficacy, and safety are guaranteed. Having resort to risk-based approaches such as Quality by Design (QbD) is much encouraged by the Food and Drug Administration and other regulatory authorities (Yu LX et al., 2014). One of the objectives of the QbD is to define a design space in which the product quality is guaranteed. This design space can be achieved with a Design of Experiment (DoE) based optimization, which helps identifying the optimal conditions as well as critical process parameters.

In this purpose, the process development department of Clean Cells has worked on implementing DoE for upstream process development (USP). DoE is an efficient statistical tool which brings knowledge on the impact of the process parameters on the responses with as few resources as possible (Bowden et al., 2019). While traditional development consists in evaluating individually each process parameter, DoE approach tests multiple combinations of these parameters together. This methodology gives therefore much information regarding the interactions between all studied parameters, the robustness of the process and its repeatability.

The objectives of the establishment of a general methodology for USP development at Clean Cells using DoE approach were as follows:

- To reach the highest phage titers within a reduced time frame
- To gain knowledge on robustness and repeatability
- To be able to select the most interesting condition depending on any specific constraints (technical, cost, robustness, time)
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MATERIAL & METHOD

The experiments described in this paper were carried out with the bacterium Escherichia coli DSM 613, and the bacteriophages T7 (DSM 4623) and T4 (DSM 4505). These bacteriophages are from different families: T7 is a *Podoviridae* while T4 is a *Myoviridae*. Cultures to be infected were thawed in the morning, grown in an incubator shaker at +37°C to reach an optical density (OD_{600nm}) of 2, then diluted to the targeted OD_{600nm} for infection. 125 mL shake flasks were inoculated, supplemented, and infected to fit the conditions instructed by the design. The media used in this study were animal-component free.

Bacteriophage titers were determined using double-agar layer method (E. Kutter, 2009). For this purpose, 5 µL drops of each harvest were spotted at multiple dilutions on a soft agar layer mixed with an *E. coli* overnight culture.

The DoEs were established with the software Design-Expert®, from Stat-Ease. For both phages, an I-Optimal Split-Splot Response Surface design was built. The advantage of the response surface method is to allow studying the effect of the interactions of the parameters with each other. This design was customed so that it could not only screen the critical parameters, but also optimize the response with a minimum number of runs. This design needed to be applicable to any other phage, so it was chosen to investigate a broad range of values for the few most interesting parameters selected. For both phages, the parameters to be tested were the Multiplicity of Infection (MOI), the bacterial OD_{600nm} at infection, the agitation during phage propagation, and the concentration of the selected culture media supplements (Supp. 1, Supp. 2, Supp. 3 for T7, and Supp. 2 only for T4). Figure 1 shows the distribution of

all conditions tested for T7 DoE depending on the supplementation solution concentrations.

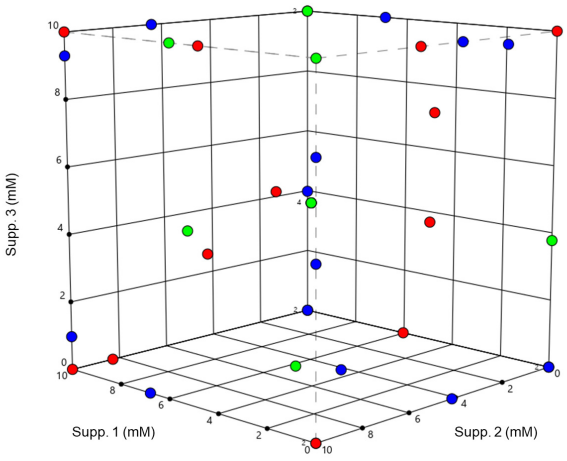


Figure 1: Scatter plot of a few parameters of T7 DoE

The parameters shown here are the supplementation solutions only. The points are colored by agitation (from the lowest in blue to the highest in red)

RESULTS

• T7 analysis

DoE and model construction:

40 conditions were tested for the optimization of T7 production process using a DoE approach. Once the harvest titers were measured, a model was created with automatic selection of significant parameters using p-values criterion. The resulting model has an adjusted R^2 of 0.96, and a standard deviation of 0.16 log_{10}PFU/mL.

Model analysis:

The first objective of this DoE was entirely focused on improving the final titer. The software's optimization tool was used with the unique constraint of the obtention of a high titer. The desirability function, which is the combination of all optimization goals into one function (ranging from zero outside of the limits to one at the optimum), is therefore identical to the titer evolution. The resulting model shows that the OD at infection has the most influence on the final phage titer (results not shown). Nevertheless, the supplementation solutions are not to be ignored as they seem to also play a role in contributing to improve the final titer. The MOI does not seem to have a significant impact on the titer.

In adequacy with the process, several constraints could also be taken into account such as low ODs at infection to limit bacterial debris, hence for an increase of clarification performances, low MOI in perspective of scale-up, and low supplementation for cheaper and simpler process. Appendix 1 represents the impact of such constraints on the desirability.

Model validation before use for prediction:

Considering all these parameters, two conditions were chosen to validate the model. These conditions are called the confirmation runs as their goal is to check that the model can rightly predict the response, in this case the titer. These conditions were the optima of two different scenarios:

- Scenario 1: Its purpose is to facilitate the scale-up of the process, hence by privileging low MOIs, low supplementation to limit costs, and by choosing a rather high OD at infection for robustness matters. Indeed, the T7 phage titer is much more robust at higher ODs where small variations of this parameter do not affect the response.
- Scenario 2: Its purpose is to diminish as much as possible the OD at infection to limit cell debris, while still achieving a high titer.

Table 1 sums up the results of these confirmation runs.

Table 1: confirmation runs data summary

		PREDICTION				RESPONSE DATA	
Scenario	Response	Predicted Mean	Total Standard Deviation	95% PI low	95% PI high	n	Data Mean
1	Titer (log_{10}PFU/mL)	**10.9**	0.2	10.7	11.2	5	**11.0**
2	Titer (log_{10}PFU/mL)	**11.3**	0.2	11.1	11.6	5	**11.2**

PI: prediction interval. n: number of runs

For each confirmation run, the data mean is very similar to the predicted one. This model can therefore be applied to predict any titer value within the experimental space.
Using this DoE based optimization, T7 phage titer shifted from a basic process result of 9.5

log_{10}PFU/mL to an optimized titer of 11.2 log_{10} PFU/mL.

• T4 analysis

DoE and model construction:

This methodology was then applied to the phage T4 as a mean to validate the previous DoE based optimization and ability to predict. However, change was made to consider a specificity of T4 process. In fact, it had been observed in preliminary experiments that the OD on T4 harvest was quite high compared to T7. It was therefore considered to be a critical parameter for DSP purpose, especially for the clarification step. This time not only the final titer was analyzed as a response, but also the OD_{600nm} of the harvest. The model was therefore adapted so that it could take into account this new response while still ensuring a good prediction capacity.

Finally, 51 conditions to be experimentally tested were obtained, split into 5 runs. As for T7, a model was then built using the data gathered from the 51 conditions.

The R^2 adjusted of the model describing the titer was of 0.88, which was lower than for T7, but still acceptable. The standard deviation of the response was estimated at 0.3 log_{10}PFU/mL. The harvest OD's model presented an R^2 adjusted of 0.97 and a standard deviation of 0.26.

Model analysis:

Appendix 2 describes the evolution of both responses depending on the factor's values. The bacterial OD for infection still shows the greatest impact on the titer but is also directly linked to a high OD of the harvest. The agitation plays a role as well on the final OD when its value increases. Finally, a supplementation with Supp. 2 seems to help improving the final titer.

Model validation before use for prediction:

Such as for T7, the model could then be exploited using different scenarios. Three conditions raised a particular interest:

- Scenario 1: Maximizing the titer while minimizing the OD of the harvest, with the same weight put on both constraints.
- Scenario 2: Maximizing the titer while minimizing the OD of the harvest, with a greater weight put on the first goal. A low MOI was also required.
- Scenario 3: Maximizing the final titer without any supplementation to lower costs of scale-up and industrialization.
- Scenario 4: Maximizing the titer exclusively

The following tables sum up the results of the confirmation runs from scenario 1 to 3 in terms of final titer and final OD. It should be noted that the scenario #4 aiming exclusively at optimizing the final titer was not carried out in the context of the confirmation runs since it was found to be precisely one of the conditions of the DoE. An average titer of 11.2 log_{10}PFU/mL was thus obtained, with a final OD of 1.35 (n=14).

Table 2: confirmation runs T4 titer data summary

		PREDICTION				RESPONSE DATA	
Scenario	Response	Predicted Mean	Total Standard Deviation	95% PI low	95% PI high	n	Data Mean
1	Titer (log_{10}PFU/mL)	**10.0**	0.3	9.5	10.5	4	**9.5**
2	Titer (log_{10}PFU/mL)	**10.3**	0.3	9.8	10.7	4	**10.4**
3	Titer (log_{10}PFU/mL)	**10.8**	0.3	10.4	11.2	4	**10.8**

PI: prediction interval. n: number of runs

Table 3: Confirmation runs OD600nm at harvest data summary

		PREDICTION				RESPONSE DATA	
Scenario	Response	Predicted Mean	Total Standard Deviation	95% PI low	95% PI high	n	Data Mean
1	OD_{600nm}	**0.03**	0.02	0.01	0.06	4	**0.01**
2	OD_{600nm}	**0.14**	0.09	0.05	0.26	4	**0.21**
3	OD_{600nm}	**1.05**	0.68	0.40	1.93	4	**0.97**

PI: prediction interval. n: number of runs

The responses of the confirmation runs were found to be within the prediction intervals of all three conditions. This model is therefore validated and seems to be able to predict any T4 titer value within the experimental space.
Using this DoE based optimization, T4 phage

titer shifted from a basic process result of 7.9 log_{10}PFU/mL to an optimized titer of 11.2 log_{10}PFU/mL.

CONCLUSION

A DoE methodology was tested by the Process Development department of Clean Cells to offer a fast, robust, and efficient phage development platform. As phages respond differently to specific culture conditions, this DoE needed to be robust and adaptable to each specificity. For each phage, these experiments were carried out in a few days, followed by a week of analysis and a final run of model validation. It would have taken weeks to reach the same productivity increase using step-by-step development, as the analytical and preparation time between experiments significantly increased the duration of the development process. What's more, there would still not be any data regarding interactions between parameters.

This approach was tested on T7 and T4 bacteriophages, both specific to *E. coli*. An improvement of the final titer was achieved on both phages, with an increase from 9.5 to 11.2 log_{10}PFU/mL and 7.9 to 11.2 log_{10}PFU/mL respectively. A design space for each phage was defined in which the phage production appeared to be robust and the phage quality guaranteed. With this data, the scale-up strategy can be secured as well as the manufacturing process.

The capabilities of the models to predict the phage titers were proven to be effective, which indicates that this approach can be used for further projects and applications. It was shown in this paper that the advantages of the DoE based optimization are numerous: not only did it allow to predict and maximize the phage titers depending on any given constraint, but also to analyze and define the robustness of the process. A solid understanding of the evolution of the titer depending on the variation of each critical parameter within the experimental space was thereby obtained.

REFERENCES

Dufour N, Debarbieux L. (2017). La phagothérapie - Une arme crédible face à l'antibiorésistance [Phage therapy: a realistic weapon against multidrug resistant bacteria]. *Med Sci (Paris)*. 33(4):410-416.

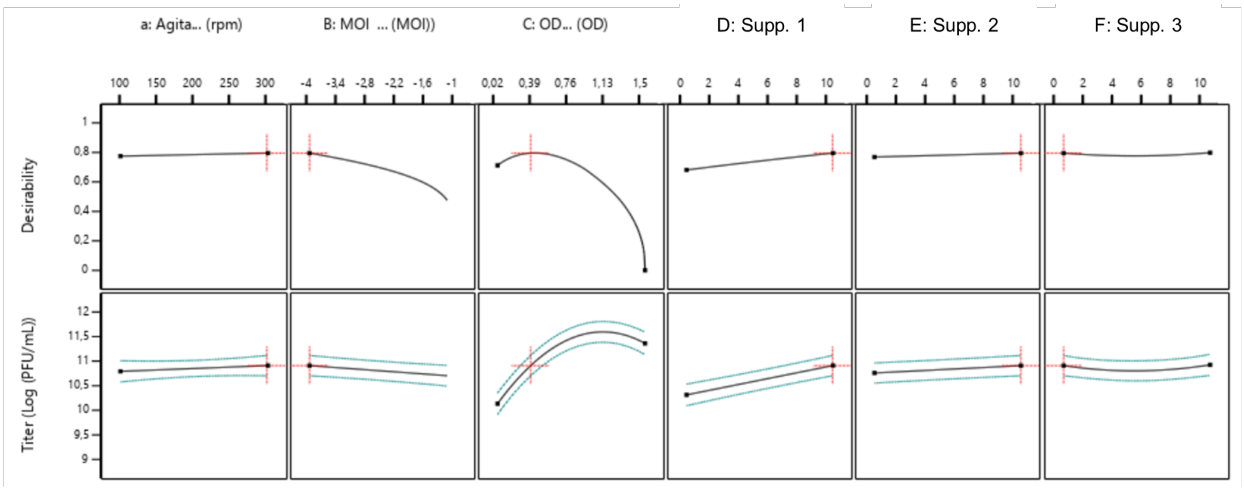
Górski A, Borysowski J, Międzybrodzki R. (2020). Phage Therapy: Towards a Successful Clinical Trial. *Antibiotics (Basel)*. 9(11):827.

Yu LX, Amidon G, Khan MA, et al. Understanding pharmaceutical quality by design. *AAPS J.* 2014;16(4):771-783.

Bowden, G.D., Pichler, B.J. & Maurer, A. (2019). A Design of Experiments (DoE) Approach Accelerates the Optimization of Copper-Mediated 18F-Fluorination Reactions of Arylstannanes. *Sci Rep 9,* 11370.

Kutter E. (2009) Phage Host Range and Efficiency of Plating. In: Clokie M.R., Kropinski A.M. (eds) Bacteriophages. Methods in Molecular Biology™, vol 501. *Humana Press.*

Appendix 1: Optimization of T7 titer using DoE analysis with multiple constraints



The red cross represents the parameters values selected for Scenario n° 2

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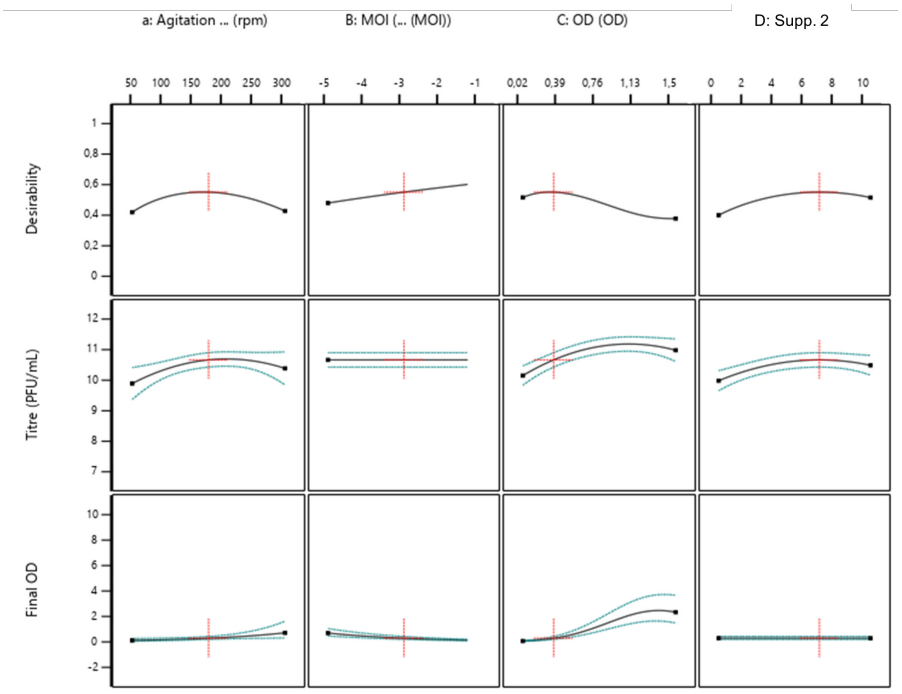
Appendix 2: Optimization of T4 titer using DoE



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