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Enhancement of the Culturing Efficacy of the HeLa Cells: An Optimization-Based Approach

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ABSTRACT
The objective of this study is the optimization of the process of culturing HeLa cells, which are a commonly used type of human cancer cell line in biological research. Furthermore, this study alsodiscusses the typical challenges faced while maintaining a successful HeLa cell culture and also shed insights into the various factors which affect this process. The study presents an in-depth analysis of the optimization process using Response Surface Methodology (RSM) as a tool, for identifying the key factors affecting cell growth and survival. The results obtained from the optimization are evaluated and it is demonstrated that the optimized process leads to improved cell yields and reduced consumption of raw materials. The findings of this study have practical implications for the broader scientific community and demonstrate the importance of process optimization in cell culture which in turn depicts the novelty of the study.

Keywords: Process optimization, Confluence, Response Surface Methodology, Box Behnken Design, D-optimality. HeLa cell line.

I. INTRODUCTION

A cell line is a population of cells that are derived from a single cell and are able to divide indefinitely. Cell lines are used in scientific research to study the properties and behavior of cells in culture(Bongso & Richards, 2004). Cell lines are useful because they allow researchers to study cells in a controlled environment, without the complexity of the whole organism. Cell lines are often established from primary cells, which are cells that are directly isolated from a tissue or organ. The cells are then cultured in the laboratory and are kept alive by being provided with the nutrients and conditions they need to survive and grow(Unchern, 1999). Some examples of cell lines include HeLa cells, which are derived from cervical cancer, and NIH/3T3 cells, which are derived from fibroblasts(Danihelová et al., 2013; Su et al. 2022). In this study, the HeLa cell line has been focussed upon. The HeLa cell line has contributed significantly to our understanding of biology and medicine, and it has played a key role in many important scientific discoveries.

The HeLa cell line is a cell line that was derived from cervical cancer cells taken from a patient named Henrietta Lacks in 1951. The cells were taken without her knowledge or consent, and they were found to have the unique property of being able to grow and divide indefinitely in culture(Lyapun et al., 2019). This made them valuable for scientific research, and they have been used extensively in laboratories around the world for more than 70 years. HeLa cells have been used to study a wide variety of biological processes, including cell growth, differentiation, and the response to stress. They have also been used to test the effects of drugs and other chemical compounds, and to study the genetics of cancer and other diseases. For using the cell lines, they need to be maintained as there are many external factors that may contaminate the cell culture (Roy et al. 2022; Song et al. 2023).

Experiments can be affected by contamination and other conditions that adversely affect cell viability (Ghoshal et al. 2022; Parveen et al. 2023). Due to the common practice of splitting cells, there is ample published evidence of cross-contamination of previously unidentified cell lines. Identifying both common and rare reasons why cells do not proliferate can improve laboratory efficiency, increase cell product yields, and provide meaningful and reliable downstream data from in vitro models(Sigma-Aldrich, 2022a). The different ways that a cell culture maybe contaminated has been tabulated below.

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Table 1 Different types of cell culture contamination

Type of Contamination	Description
Cell line Misidentification	Up to one third of all cell lines are affected due to cell line misidentification. Contamination can be due to invasion of aggressive cell lines, such as HeLa cell line or because of sharing cells between laboratories and causes errors in cell line identification.
Microbial Contamination of Cell Cultures	Cell culture media and the controlled environment of the laboratories provide an ideal setting for bacterial, fungal and viral contaminants. Strict sterilization techniques must be performed on a regular basis for signs of microbial invasion (Sigma-Aldrich, 2022a). However, continued usage of antibiotics promotes the development of antibiotic-resistant strains. Antibiotics can sustain low levels of contamination but can result into full-blown contamination when they are removed from the medium (Thermo Fisher Scientific).
Precipitates in Culture Media	When the cell culture media show turbidity despite having no contamination, it is usually due to precipitation of metals, proteins and other components of the medium (Sigma-Aldrich, 2022b).

However, it is possible to keep a cell culture environment free of contaminants and improve the quality of the cells being cultivated by employing the proper tools and methods. Cell Culture, especially mammalian cell culture, is a technique that mimics the physiological conditions in a laboratory growth medium to ensure the healthy growth of cells in anin-vitro environment. There are a number of important physical elements that affect the growth and upkeep of cell cultures, including:

Temperature: The temperature of the cell lines must be adjusted to the internal environment of the organisms. The optimal temperature for human and mammalian cell lines is 36-37℃, while it is much lower at 15-26℃ for cold-blooded animals(Segeritz & Vallier, 2017). Increasing temperature can cause certain proteins to denature, while decreasing temperature can slow down polypeptide catalysis and initiation(Sartorius, 2023).

pH: The pH of most laboratory-cultured human and mammalian cell lines must be tightly controlled and maintained at physiological pH levels which is between 7.2 and 7.4. A few fibroblast cell lines proliferate at moderately alkaline conditions between 7.4 and 7.7, whereas transformed cells grow in a relatively acidic medium between 7.0 and 7.4(Segeritz & Vallier, 2017). Variance in intracellular pH can affect all cellular processes, like metabolism and cell growth(Robert W. Putnam, 2012). The equilibrium between CO_2 and HCO^{3-} determine the pH levels. Hence, pH buffers are added to adjust the CO_2 concentrations(Segeritz & Vallier, 2017).

CO_2 concentration: Variation in the concentration of atmospheric CO_2 can also change the pH. Hence, cell cultures are grown in incubators that regulate carbon dioxide concentrations between 5 and 7%(Segeritz & Vallier, 2017). Persistent exposure to high concentrations of CO_2 leads to reduced cell growth and mitochondrial dysfunction(Vohwinkel et al., 2011).

O_2 concentration: In mammalian bodies, oxygen levels vary from 1 to 12%, whereas it is 21% in the atmosphere. In a hypoxic environment, cells grow faster, live longer and are less stressed. These conditions can be achieved using incubators that provide nitrogen, in addition to carbon dioxide(Bates, 2012).

Process Optimization

Process optimization is the improvement of a particular process in order to increase efficiency, reduce costs, improve quality, or achieve other desired outcomes. It is an important tool that ensures that process designs remain economical while upholding the specifications(Atli Freyr Magnússon, 2020). The goal of process optimization is to find the best and most efficient way to perform a particular task, while also identifying and eliminating inefficiencies and bottlenecks.

There are several types of optimization tools, Artificial Neural Network (ANN), Machine Learning (ML) and Response Surface Methodology

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(RSM) etc. to name a few(Singhal et al., 2022). The former two require an in-depth knowledge and expertise for operation. They do not generate an equation so even the slightest changes would require the user to change the entire code, which is why, RSM has been selected for this study as it generates an equation that can be used in the entire factor space (Roy, Debnath, and Ray 2022; Roy and Ray 2022).

Response Surface Methodology (RSM) designs were developed by the scientists George E. P. Box and K. B. Wilson in 1951(Box, 1999). Majorly, three classes of designs – Taguchi, Central Composite Design (CCD) and Box Behnken Design (BBD) have emerged(Singh et al., 2011). Taguchi has shown high signal to noise ratio and hence the outputs have a lot of anomalies that cannot be examined without expertise (Azmi et al., 2013). CCD and BBD both explain the system's behaviour and its optimization. CCD can be used after screening for important factors (Roy and Ray 2019; Roy and Ray 2020). It consists of central and axial points of the adjoining cube points and illustrates the curvature of the response and determines higher-order effects. BBD shares similarities with CCD but needs a smaller number of trial runs and excludes the cube's points at the vertices, that is, excludes the extremities(Jankovic et al., 2021). For three or more factors in a quadratic response surface model, BBD offers some advantage as it requires fewer number of runs and has higher precision and the results tend to be less biased(Roy, 2022). Further, BBD ensures that not all factors are set to their highest levels simultaneously. Hence, BBD was selected for this particular study.

The main objective of this study is to optimize the process parameters to produce high-quality products in a cost-effective and sustainable manner. It has made use of statistical design of experiments to identify the key variables that affect process performance and to optimize their values. This type of optimization for HeLa cell culture has not been reported in the literature, as per author's best knowledge.

Table 2A summary of the previously conducted works collected from the literature in this particular domain (2017-2022).

S. No.	Major Findings	Reference
1	• During the COVID-19 outbreak, HeLa cells were used to test the infectivity of the virus. • However, the coronavirus did not infect the HeLa cells effectively. • It was found that these cells lacked the ACE2 molecules, that the virus used to enter the cells. • Then the HeLa cells were genetically engineered to accommodate the ACE2 molecules. • Now, the virus could enter the cells and infect it. • Thus, using the HeLa cells, the entrance for SARS-CoV-2019 particles was recognized and further research about its transmissibility could be carried out.	(Zhou et al., 2020)
2	• According to the study, the HeLa cells that had been procured from a common source showed a considerable amount of copy number variation (CNV) in different laboratories after they have been sub-cultured a few times. • Despite HeLa cells being immortal, it has been found that the results are inconsistent if different generations of the cells are used.	(Liu et al., 2019)
3	• Silver nanoparticles capped with glucose have the ability to induce arrest of the cell cycle in HeLa cells. • The G2/M phase of the cell cycle was majorly affected, although it influenced the whole	(Panzarini et al., 2017)

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	cell.	
4	• This study illustrates the anti-tumor activity exhibited by Lactobacillus paracasei present in human breast milk. • They showed inhibition rates of up to 89% by a particular strain of the bacteria.	(Rajoka et al., 2018)
5	• In this study, the tumor cells were targeted at by a combination of transferrin conjugated liposome (Tf-PEG) and ultrasound (low-frequency). • The liposomes were loaded with calsein, a fluorescent dye, to visualise the Tf-PEG lysosome uptake by HeLa Cells. • It showed an increase in uptake compared to the normal lysosome by 151%. • Hence, it is effective as a drug delivery system.	(AlSawaftah et al., 2021)

II. MATERIALS AND METHODS

Cell culture

The HeLa cells, procured from American Type Culture Collection (ATCC), were cultivated in Dulbecco's Modified Eagle Medium (DMEM) manufactured byThermo Fisher Scientific Inc., complemented with 100 µg/mL of streptomycin.

DMEM is a nutrient media frequently used for culturing mammalian cells, including the Hela cells. In some experimental set-ups, Eagle's Minimal Essential Medium (EMEM) is also used. However, it has been found that the amino acid content in DMEM is almost four times that of EMEM. The media also contains other essential nutrients such as vitamins, minerals and glucose as well as a growth factor - Foetal Bovine Serum (FBS) (Thermo Fisher Scientific Inc.). The concentration of FBS in DMEM is commonly around 10%.

The medium must also contain antibiotics such as penicillin and/or streptomycin. While antibiotics are essential in subculturing to prevent microbial contamination, their over usage can lead to the growth of antibiotic resistant strains(Russell, 2003). The antibiotics can be added to the media directly or separately to the cells after they have undergone trypsinization and reseeded into new flasks. The concentration of the antibiotics used should be checked with the recommendation of the supplier, or this case according to the levels prescribed by ATCC.

For subculturing of the HeLa cells, a CO_2 incubator had been used. These types of incubators can regulate the CO_2 concentration, which is typically around 5% in physiological conditions, the concentration of CO_2 helps to maintain the desired pH of the culture medium, which is around 7.4 and obviously the temperature, which is around 37°C. They also have a gas control system to maintain the maintain the accurate concentration of O_2 and as well as the humidity required for cell growth and metabolism(Segeritz & Vallier, 2017). In addition, the incubators need to be calibrated properly for preventing the growth of any contaminants (Dey et al. 2022;Roy et al. 2022).

When the cells reach confluency, they are trypsinized, and sub-cultured according to the cell type and passage number at a ratio of 1:3 to 1:10 into a fresh medium. The cells must be examined at regular intervals for any signs of contamination or cell death(Sandell & Sakai, 2011). These signs can be morphological changes or growth of microorganisms.

III. RESULTS AND DISCUSSION

Selection of Process Parameters and Model Development

The most important factorsidentified for mammalian cell culture are nutrient supply, carbon dioxide levels, pH of the media, temperature, humidityand oxygen levels (ibidi GmbH, 2023). The next step, through literature survey, was to determine the ones that have significant contribution towards cell growth and maintenance.Temperature, carbon dioxide and pH (of the media) were ascertained to be the most significant ones and were considered for the development of the RSM model. The level of these three factors for the experimental design is presented in Table 3. The experimental design adopted for this study has three factors at three levels (Vipparla et al. 2022; Roy et al. 2022).

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Table 3 Factors and their levels used in the experimental design

Levels	Temperature	CO_2 conc.	pH
1	36°C	3%	6
2	37°C	4%	7
3	38°C	5%	8

The levels for each factor weredecided, as reported in the literature. The temperature limits were noted mostly between 36℃ and 38℃ (Jahagirdar et al., 2018), (Chang et al., 1961) and (McCormick & Penman, 1969). The pH range was observed from 6 to 8 (Kollmorgen & Griffin, 1969), (Trebinska-Stryjewska et al., 2020) and (Abdeen et al., 2011). And carbon dioxide was seen to be ranging from 3 to 5% (Santy et al., 2001) and (Puck & Marcus, 1955). Thereafter, an RSM-based optimization was performed by employing Box Behnken Design approach using Design-Expert® Software (Version 13) to determine the optimized process parameters for HeLa cell culture, as presented in Table 4. Confluence % was used to determine the experimental results (or process outcome) after the cell cultures had been incubated for 48 hours. The standard deviations were found to be statistically insignificant and hence have not been incorporated in the table.

Table 4 Design of Experiments adopted for the optimization of the process parameters for maximizing the response outcome

Run ID	Factor 1: Temperature (Celsius)	Factor 2: Carbon Dioxide Concentration (%)	Factor 3: pH (Units)	Response: Confluence %
1	38	3	7	68
2	38	4	8	72
3	36	4	8	70
4	36	4	6	66
5	38	5	7	82
6	37	4	7	74
7	37	4	7	72
8	37	5	6	78
9	37	4	7	86
10	36	3	7	66
11	36	5	7	64
12	38	4	6	58
13	37	3	6	78
14	37	3	8	78
15	37	5	8	76

The DOE shown in Table 4 was then used to produce a model in the form of a quadratic equation (Equation 1). Analysis of variance (ANOVA) and multiple linear regression analysis were used to create the model. The data shown in Table 4 were used to perform the ANOVA. The coefficients having p-values greater than 0.05 with a 95% confidence level were used to determine which terms were statistically significant. In Table 5, the ANOVA's findings are presented.

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Table 5 Results obtained from the analysis of ANOVA conducted with the design of experiments

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	1085.31	1	1085.31			
Linear vs Mean	0.2429	3	0.0810	0.3637	0.7805	
2FI vs Linear	0.3192	3	0.1064	0.3998	0.7571	
Quadratic vs 2FI	1.19	3	0.3976	2.12	0.2160	Suggested
Cubic vs Quadratic	0.5747	3	0.1916	1.06	0.5194	Aliased
Residual	0.3619	2	0.1810			
Total	1088.00	15	72.53			

The replicates of the blocks' statistical insignificance indicated that the data were uniform. The ANOVA findings showed that the generated model included statistically significant interaction, linear and quadratic terminologies in it. The lack of fit demonstrated the model's error contribution. As a result, a complete quadratic equational model was established utilising the ANOVA findings. The final model equation, which is shown in Equation 1, only took into account the statistically significant elements.

Equation 1

Square Root (Confluence) = -667.98003 +38.25062 (Temperature) -9.26010 (CO_2 Concentration) -4.21450 (pH) +0.233303 (Temperature * CO_2 Concentration) +0.156737 (Temperature * pH) -0.028491 (CO_2 Concentration * pH) -0.543007 (Temperature^2) +0.112340 (CO_2 Concentration^2) -0.096145 (pH^2)

Equation 1 was used to determine the confluence % at various factor-setting levels in order to assess the created model's prediction accuracy. Then, the model predicted data was plotted against the experimentally determined data, presented in Figure 2. The computed results suggested that the correlation coefficient (R^2) obtained between experimentally determined and model-predicted response is 0.6520, which implies a good fit. Additionally, the best fit curve showed that all of the data points fell inside the 95% confidence level. As a result, it was concluded that the constructed model's prediction accuracy was suitable for further optimization and the detection of factor effects.

Model Validation

The model predicted data was compared to the experimental data. The verification of the model was done by accessing the difference between the model predicted and experimentally determined response at the same factor levels (Roy, 2022). The normality plot shows a near straight line with most of the points lying very close to or on the curve which in turn indicated that the data is normally distributed (Oppong & Agbedra, 2016).

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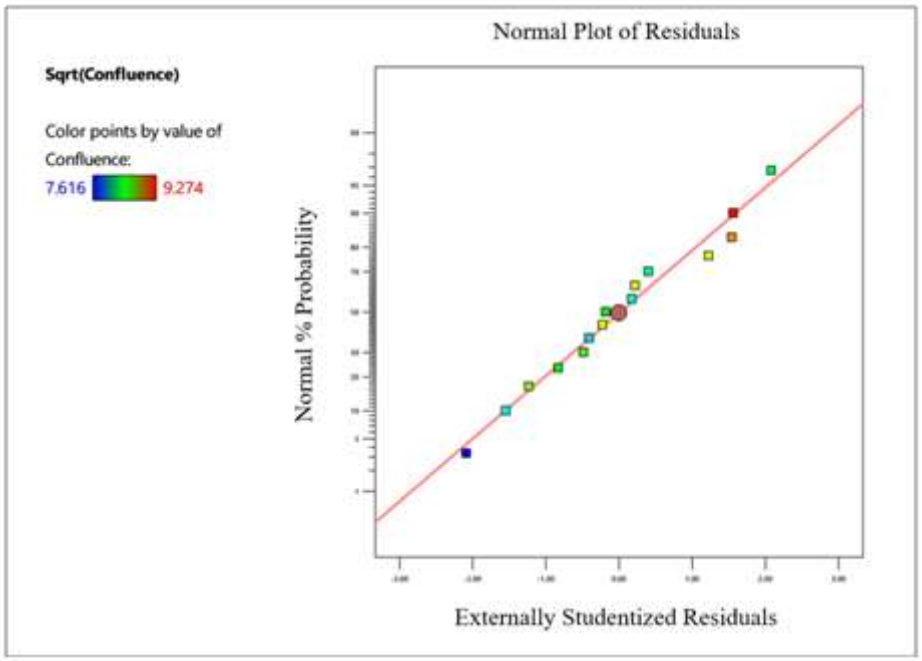

Figure 1 Normality Plot.

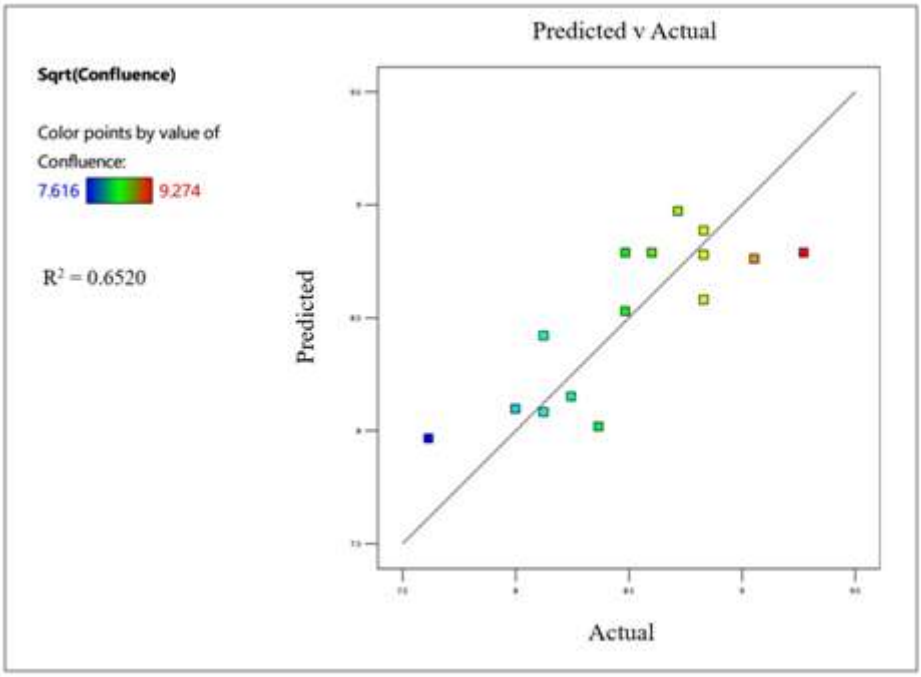

Figure 2 Evaluation of the accuracy of the developed model through the plot between the experimental and model-predicted data.

The factor setting for boosting cell growth (confluence%) was identified using the established response surface model. To identify the factor level setting for optimising the output, three-dimensional response surface plots developed by the software. Figure 3 (A-C) show the results that were achieved. In Figure 3(A) when CO_2 and temperature were plotted against the confluence, it can be observed that the confluence increases in the beginning, then peaks and gradually declines. A similar trend can be seen in Figure 3(B) where, pH and temperature have been taken. The confluence again increases

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with the increase in pH and temperature peaks and then declines. However, in Figure 3(C), when pH and CO_2 concentration were taken, not much interaction is noted.

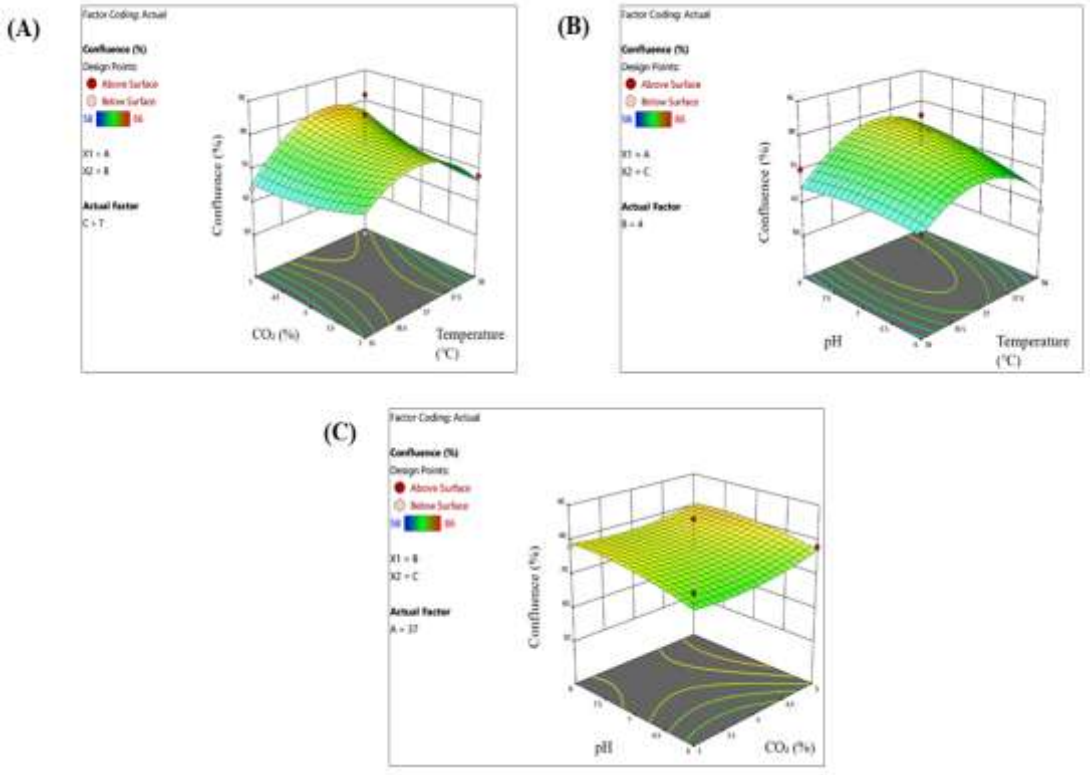

Figure 3 Response Surface Plots of different process parameters on the cell culture (A) Synergistic Effect of CO_2 and Temperature on Confluence %; (B) Synergistic Effect of pH and Temperature on Confluence %; (C) Synergistic Effect of pH and CO_2 on Confluence %.

In order to identify the optimal factor setting that corresponds to the maximum confluence %, a numerical optimization was ultimately carried out. D-optimality, which is one of the most used tools for numerical optimization, was chosen to complete this task. The value of D-optimality typically ranges from 0 to 1, with 0 denoting the worst conditions and 1 denoting the one that is optimum(Zhang et al., 2018). The software used in this study projected a number of D-optimality solutions. The best combination among them was selected and has been shown in Table 5 below.

Table 5 Numerical Optimization of the process parameters for HeLa Cell Culture by adopting D-optimality

Temperature	CO2 Conc.	pH	Confluence	Desirability
37.256	4.896	7.335	81.554	1.000

According to the information in Table 5, temperature 37.256, 4.896% CO_2, and 7.335 pH were the ideal process parameters for maximizing the reaction outcome (expressed as confluence %). Confluence % at this specific factor setting was found to be 18.554%, as predicted by the model.

IV. CONCLUSION

The present study showcased a novel approach to optimize the process parameters corresponding to defining the best condition for culturing HeLa cell line. Among numnerous literature reported factors, the pH, CO_2 concentration, and incubation temperature was shortlisted. The confluence (in terms of percentage) was considered as the process response output. A full quadratic model was obtained to predict the process outcome within the defined factor space. The 3-dimensional response surface plots indicated the impact of the various process parameters on the response outcome. After the validation, it was found that the experimentally determined response

were in sync with the model predicted outcomes which inturn defines the acceptance of the developed model. The future scope of process optimization is vast and continually evolving as new technologies and techniques become available. The trend in process optimization is toward more data-driven, automated, and intelligent approaches that can continuously improve processes over time. This study presents the opportunity for further research on the matter in the future, which could not be performed now due to time and resource constraints. The factors can be selected by running a Principal Component Analysis (PCA). Usage of enhanced models such as Artificial Neural Network (ANN) and Machine Learning (ML) can be made use of. The factor levels that have been collected by studying the literature can be validated experimentally.

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