JAP&T
TPA-Timişoara

Available online at http://journal-of-agroalimentary.ro

Journal of Agroalimentary Processes and Technologies 2013, 19(2), 185-193

Journal of Agroalimentary Processes and Technologies

Modelling the destruction of *Saccharomyces cerevisiae* and *Candida utilis* yeast cells using Intense Pulsed Light

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Received: 30 May 2013; Accepted: 25 June 2013

Abstract
Experimental tests were performed to evaluate the effect of intense pulsed light (IPL) treatment on microbial inactivation of yeast cell suspensions of *Saccharomyces cerevisiae* CBS 493.94 and *Candida utilis* MIUG 3.5. Regression analysis of microbial inactivation with IPL was then investigated. The aim of this study was to apply the response surface methodology (RSM) to define the relationship between the reduction of yeast cells and the process parameters, namely energetic density of light and duration of IPL treatment. Moreover, RSM was used to verify the predicted models and to adapt them on particular conditions of yeast cells inactivation by IPL treatment. The final model shows the dependency of the yeast cells reduction by independent variables (energetic density of light and duration of IPL treatment) and certain binary interactions of the IPL process parameters. The response surface follows the reduction of yeast cells and reaches the lowest level for $N/No = 0$. This level represents the optimal area of the response surface, which is ideal for any microbial inactivation treatment.

Keywords: Modelling, Pulsed Light, response surface methodology, *Saccharomyces cerevisiae*, *Candida utilis*, yeast, inactivation.

1. Introduction

Intense pulsed light (IPL) is an emerging non-thermal technique that involves the use of a clear fused quarts tube filled with xenon able to produce short pulses of intense broad-spectrum of electromagnetic radiation [1-6]. This spectrum is very similar to sunlight and contains wavelengths from 200 nm in the ultraviolet (UV) to about 1000 nm in the near-infrared region. It has peak emission between 400 and 500 nm and contains some UV wavelengths that are missing in the sunlight because they are filtered by the earth's atmosphere. Each PL flash has an intensity of approximately 20,000 times that of sunlight at sea level [7].

There are some applications in which IPL may be used as an alternative to conventional heat treatment for food and material surfaces decontamination by destroying microbial population, both vegetative cells and spores.

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The ability of microorganisms' inactivation has been demonstrated in many studies: *Bacillus* sp. on wheat flour and black pepper [8], *Botrytis cinerea* and *Monilia fructigena* on strawberries [9], *E. coli* and *Listeria innocua* on fresh-cut mushrooms [10], *Listeria monocytogenes* on ready-to-eat (RTE) sausages [11], infant foods [12], and RTE cooked meat products [13], native microbiota of fresh-cut mushrooms [14], *Salmonella enteritidis* on shell eggs [15-18], *L. monocytogenes* and *E. coli* on meat slicing knife [19], *L. innocua* on stainless-steel surfaces [20-21], *Aspergillus niger*, *A. cinnamomeus* and *Cladosporium herbarum* on paper–polyethylene packaging material [22].

The decrease in microbial concentration has been explained in several mathematical models, linear or non-linear. Thus, linear models related the surviving microorganisms or the microbial survival fraction, which is defined as the ratio of the living cells before and after treatment (N/No) with time [22] or with the

main characteristic of the applied treatment, e.g. electric field strength [23, 24] for pulsed electric field (PEF) or energetic density of light [22, 25].

Many authors suggested a nonlinear relationship between the logarithm of the survival fraction and the electric field strength [26], energetic density or light fluence [11] or pressure [27] for high hydrostatic pressure (HHP). In 1997, Martín-Belloso et al. assumed the first-order kinetics by considering the exponential decay behaviour of the *Escherichia coli* survival fraction with treatment time [28]. One of the most used nonlinear models is the Weibull model [29], introduced as an alternative for the classical Bigelow model of first-order kinetic. Weibull model takes into account the biological variation, with respect to thermal or non-thermal inactivation and is basically a statistical model of distribution of inactivation times. It is widely used to explain the inactivation of microorganisms using different treatments e.g. PEF [30, 31] or PL [32-34] or simply the survival of microorganisms on different substrates [35].

The average values obtained for microbial inactivation under traditional experiments with given working conditions do not provide any information of whether or not the substrate has been uniformly treated. A non-uniformly treated food is undesirable and potentially unsafe for consumption [33]. Therefore, it is important to improve the performance of treatments using a method of optimization. Usually, in practice one parameter is changed while the others are kept at a constant level. The major disadvantage of this one-variable-at-a-time technique is that it does not include interactive effects among the variables. To overcome this problem, optimization studies can be done using response surface methodology (RSM) [36].

RSM consists of a group of mathematical and statistical techniques that can be used to define the relationships between the response and the independent variables. RSM defines the effect of the independent variables, alone or in combination, on the processes. In addition to analyzing the effects of the independent variables, this experimental methodology generates a mathematical model. The graphical perspective of the mathematical model has led to the term Response Surface Methodology [36, 37].

The purpose of this study was to apply the RSM to the inactivation with IPL of two yeasts, *Saccharomyces cerevisiae* CBS 493.94 and *Candida utilis* MIUG 3.5, in order to verify predicted models and to adapt them on particular conditions of microorganisms' growth.

2. Materials and Methods

2.1. Materials

2.1.1. Yeast cultures

S. cerevisiae strain used in this study is CBS 493.94, a brewery yeast isolated from the commercial live yeast culture Yea-Sacc®1026 (Alltech-SUA), a feed supplement containing 5×10^6 CFU/g. Isolation was performed on Malt Extract Agar (Scharlau, Spain).

C. utilis MIUG 3.5 was kindly provided by the Microbiology Laboratory of the *Dunarea de Jos* University of Galati.

Yeast pure cultures were kept at –80°C in glycerol and reactivated on Malt Extract Agar (Scharlau, Spain) when needed.

2.1.2. Pulsed light equipment

Pulsed light has been produced using an experimental set-up equipped with a power supply unit and a chamber in which a flash lamp is fixed on top. The power supply unit includes an energy storage capacitor which accumulates the electrical energy multiplying it many folds, than releases this energy in a very short time as a very short light pulse in the flash lamp.

The flash lamp used was IFP-800 type with discharge through xenon gas (wavelength of electromagnetic field, λ = 200 – 1000 nm; pulsed light regime, $t = 10^{-3}$ s; light intensity, E = 1000 – 8000 J).

2.2. Methods

2.2.1. Inocula preparation

Cells used in the experiments were obtained by cultivating the yeasts pure cultures on slants of Malt Extract Agar (Scharlau, Spain), for 5 days at 25°C. The biomass was cropped and suspended in 0.8 % NaCl solution. Cells were enumerated using a haemocytometer (Thoma). Suspensions containing $10^4 – 10^5$ cells/mL were used for sample preparation.

2.2.2. Sample preparation

Volumes of 10 cm^3 from yeast suspensions were poured in sterile Petri dishes (Φ = 100 mm) and gently rotated to generate a thin layer on the dish

surface. Then, dishes were submitted to IPL treatments.

2.2.3. Treatment with IPL

Petri dishes were placed uncovered on the support inside the treatment chamber at a distance of 7 cm from the flash lamp and treated with IPL, excepting the control samples.

Applying electric voltages of 600 V, 880 V, 1035 V, 1200 V and 1350 V, energetic densities of 0.18, 0.36, 0.54, 0.72 and 0.90 J/cm^2/pulse were obtained. Such treatments were performed for 2, 4, 6, 8 and 10 pulses, therefore total energetic density varied from 0.36 J/cm^2 (0.18 J/cm^2/pulse × 2 pulses with duration of 10^{-3} s each) to 9.0 J/cm^2 (0.90 J/cm^2/pulse × 10 pulses with duration of 10^{-3} s each).

All experiments were replicated three times in order to determine the significant and non-significant differences in log CFU/cm^2 of yeast cells subjected to each treatment.

2.2.4. Microbiological analysis

Volumes of 1 cm^3 from treated and untreated yeast suspensions were introduced in sterile Petri dishes and covered by MEA (Scharlau, Spain). Petri dishes were then incubated at 25°C for 5 days, when colonies were enumerated in order to count yeast survivors.

2.2.5. Theory and calculations

An optimizing study using RSM can be separated into three stages. The first stage, also called the selection of model dimensions stage, consists in the preliminary work performed to determine the independent parameters and their levels. The selection of the experimental design and the prediction and examination of the model equation belong to the second stage. The last stage comprises the obtaining of the response surface plot as a function of the independent parameters and determination of optimum points [36].

The preliminary work, carried as described above (points 2.2.1 – 2.2.4), provides the experimental results used to perform the RSM study. Since independent parameters have different units and/or ranges in the experimental domain, they must be normalised before performing a regression analysis. This is done by coding the parameters, forcing them to range from – 1 to 1. Commonly used equation for coding is:

$$X = \frac{x - \frac{x_{\max} + x_{\min}}{2}}{\frac{x_{\max} - x_{\min}}{2}} \quad (1)$$

where x is the natural variable, X is the coded variable and $x_{\max}$ and $x_{\min}$ are the maximum and minimum values of the natural variable [36].

The selection of the experimental design depends on the available computer packages. Then, the model equation is defined and the coefficients of the model equation are predicted. Usually, the RSM study uses a full quadratic equation or the reduced form of this equation [36]. The second order model can be written as follows:

$$y = a_o + \sum_{i=1}^{n} a_i X_i + \sum_{i=1}^{n} a_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{m} a_{ij} X_i X_j \quad (2)$$

where a_o, a_i, a_{ii} and a_{ij} are regression coefficients for intercept, linear, quadratic and interaction coefficients respectively and X_i and X_j are coded independent variables.

Written for two independent variables, equation (2) becomes:

$$y = a_o + a_1 \cdot x_1 + a_2 \cdot x_2 + a_{11} \cdot x_1^2 + a_{22} \cdot x_2^2 + a_{12} \cdot x_1 \cdot x_2 \quad (3)$$

Having the regression coefficients, the estimated response is easily calculated using the model equation. Afterwards, the examination of the model suitability with the experimental data is carried on using techniques such as residual analysis, prediction error sum of squares (PRESS) residuals (calculation of the coefficient of determination, R^2), or absolute average deviation (AAD) which is a direct method used to directly describe the deviations [36]:

$$AAD = \left\{ \left[\sum_{i=1}^{p} \left(\left| y_{i,\exp} - y_{i,cal} \right| / y_{i,\exp} \right) \right] / p \right\} \cdot 100 \quad (4)$$

where $y_{i,\exp}$ and $y_{i,\text{cal}}$ are the experimental and calculated responses, respectively, and p is the number of experimental run. Evaluation of R^2 and AAD values together should be better to check the accuracy of the model. R^2 must be close to 1.0 and the AAD between the predicted and observed data must be as small as possible [36].

The experimental design used in this study was a statistical experimental design for two independent variables called face-centred-design (FCD) whose schematic design is presented in Fig. 1. Jandel Scientific programme (Table Curve 3D) was used to perform the RSM study and to obtain the surface response plots.

3. Results and discussion

3.1. Selection of model dimensions

The inactivation of yeast cells by applying IPL treatment operates with the following variables:

- Independent variables: energetic density of light (*Ed*, J/cm^2) and time (*t*, s);
- Dependent variable (response): reduction of microbial population (*N*/*N*o) where *N*o and *N* are the numbers of living cells before and after IPL treatment, in CFU/ml).

The range and level of variables were:

- Energetic density, E_d = 0.18 – 0.9 J/cm^2/pulse (0.18, 0.36, 0.50, 0.72, 0.90 J/cm^2/pulse);
- Time, t = (2-10)×10^{-3} s (2×10^{-3}, 4×10^{-3}, 6×10^{-3}, 8×10^{-3} and 10×10^{-3} s).

Independent variables are codified using the equation (1) and the results are presented in Table 1.

Table 1. Indepdendent variables – natural and codified values

Independent variables	Symbol		Level	
	Natural	Codified	Natural	Codified
Energetic density, J/cm^2	E_d	x_1	0.18	– 1
			0.36	– 0.5
			0.54	0
			0.72	0.5
			0.90	1
Time, s	t	x_2	0.002	– 1
			0.004	– 0.5
			0.006	0
			0.008	0.5
			0.010	1

Usually, for the RSM study, at least 3^2 = 9 experiments are required in order to obtain all the possible combinations of independent variables. In this work, 5^2 = 25 combinations were applied. Those combinations and the values obtained for the dependent variables (responses) are presented in Table 2.

Table 2. Experimental matrix

Independent variables		Response, *N*/*N*o	
x_1	x_2	*S. cerevisiae* CBS 493.94	*C. utilis* MIUG 3.5
– 1	– 1	0.2373	0.3549
– 1	– 0.5	0.1374	0.1689
– 1	0	0.0391	0.0933
– 1	0.5	0.0188	0.0294
– 1	1	0.0041	0.0004
– 0.5	– 1	0.1188	0.0788
– 0.5	– 0.5	0.0309	0.0228
– 0.5	0	0.0104	0.0068
– 0.5	0.5	0.0041	0.0003
– 0.5	1	0	0.0001
0	– 1	0.0531	0.0440
0	– 0.5	0.0159	0.0008
0	0	0.0002	0.0003
0	0.5	0.0001	0.0001
0	1	0	0
0.5	– 1	0.0119	0.0025
0.5	– 0.5	0.0004	0.0001
0.5	0	0	0.0001
0.5	0.5	0	0
0.5	1	0	0
1	– 1	0.0042	0.0003
1	– 0.5	0	0
1	0	0	0
1	0.5	0	0
1	1	0	0

3.2. Selection of experimental design and model equation

The FCD experimental design selected for this study is presented in Fig. 1.

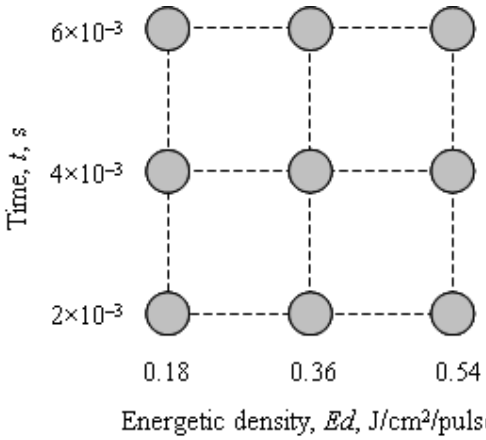

Fig. 1. Schematic of face-centered design (FCD) statistical experimental design for two independent variables.

Analysing the values of responses (*N*/*N*o) from Table 2, total inactivation is observed in several cases. Therefore, the experimental design operates with the minimum number of experiments required, meaning $3^2 = 9$ in order to avoid most of the total inactivation cases.

Table 3 presents the level of responses both for experimental and estimation, followed by the value of AAD calculated using equation (4).

Table 3. Measured and estimated responses for *S. cerevisiae* CBS 493.94 yeast cells

Independent variables		Measured response, *N*/*N*o	Estimated response using RSM
E_d, J/cm^2	Time, s		
0.18	0.002	0.2373	0.2340
0.18	0.002	0.2370	0.2390
0.18	0.002	0.2374	0.2360
0.18	0.004	0.1374	0.1370
0.18	0.004	0.1370	0.1365
0.18	0.004	0.1375	0.1360
0.18	0.006	0.0391	0.0410
0.18	0.006	0.0390	0.0400
0.18	0.006	0.0392	0.0390
0.36	0.002	0.1188	0.1100
0.36	0.002	0.1190	0.1200
0.36	0.002	0.1189	0.1180
0.36	0.004	0.0308	0.0580
0.36	0.004	0.0310	0.0320
0.36	0.004	0.0309	0.0300
0.36	0.006	0.0104	0.0190
0.36	0.006	0.0104	0.0110
0.36	0.006	0.0105	0.0100
0.54	0.002	0.0530	0.0500
0.54	0.002	0.0530	0.0520
0.54	0.002	0.0531	0.0530
0.54	0.004	0.0159	0.0157
0.54	0.004	0.0160	0.0170
0.54	0.004	0.0159	0.0150
0.54	0.006	0.0002	0.0004
0.54	0.006	0.0002	0.0003
0.54	0.006	0.0002	0.0002
AAD			**14.177**
R^2			0.97

Similar calculations were done for *C utilis* MIUG 3.5 and they revealed an AAD value of 11.713 and R^2 of 0.953. Since R^2 are closed to 1 for both yeasts and AAD values are smaller than 15% the predicted model (equation 3) accuracy is high.

Equation (3) can be rewritten as follows:

$$\frac{N}{N_o} = a_o + a_1 \cdot E_d + a_2 \cdot t + \\ + a_{11} \cdot E_d^2 + a_{22} \cdot t^2 + a_{12} \cdot E_d \cdot t \quad (5)$$

The values of all coefficients of equation (5) were obtained performing the RSM study in Jandel Scientific Table Curve 3D for *S. cerevisiae* CBS 493.94 yeast cells (Table 4) and *C.utilis* MIUG 3.5 yeast cells (Table 5).

To verify the assumptions concerning the selected models and coefficients, tests *t* and *F* were used. Both tests were calculated for a significance level $\alpha = 0.05$ (Tables 4-6). The regression analysis has the facility to select the most significant coefficients of the model equation framing in the risk level and to redefine the new models (Table 6). The selected coefficients were marked with "√" and the others, considered irrelevant for the process, were excluded from the regression equations presented in Table 6.
The particular equations presented in Table 6 are generalised in the final model:

$$\frac{N}{No} = a_o - a_1 \cdot E_d - a_2 \cdot t + a_{12} \cdot E_d \cdot t \quad (8)$$

This model shows a good proximity of functional dependencies of variables and polynomial functions, aspect illustrates by the correlation coefficients R^2 which are quite closed to 1. The response depends directly of both independent variables through the terms of equation (8) containing only the energetic density or the time. Missing terms containing the squared independent variables would indicate the simplicity of the destruction mechanism. The presence of the last term as product of independent variables shows certain binary interactions of these parameters such as: an increased energetic density of light would reduce the action time of pulsed light to obtain the same effect of cell destruction and vice-versa.

Table 4. Non-linear regression coefficients estimation for *S. cerevisiae* CBS 493.94 yeast cells

Independent variables	Coefficients Symbol	Value	Standard error	*t*-value	P >|t|	Significant coefficients
E_d	a_o	0.3857	0.032	12.037	0.00000	√
t	a_1	-0.6149	0.087	-7.063	0.00000	√
	a_2	-54.4256	7.836	-6.945	0.00000	√
	a_{11}	0.2466	0.073	3.372	0.00321	
	a_{22}	1943.9285	592.683	3.278	0.00394	
	a_{12}	39.1361	5.509	7.103	0.00000	√

Table 5. Non-linear regression coefficients estimation for *C.utilis* MIUG 3.5 yeast cells

Independent variables	Coefficients Symbol	Value	Standard error	*t*-value	P >|t|	Significant coefficients
E_d	a_o	0.5148	0.060	8.555	0.00000	√
t	a_1	-0.9873	0.163	-6.038	0.00001	√
	a_2	-62.3607	14.715	-4.237	0.00045	√
	a_{11}	0.4815	0.137	3.504	0.00237	
	a_{22}	1922.9107	1113.038	1.727	0.10028	
	a_{12}	51.9629	10.347	5.022	0.00008	√

Table 6. Correlation coefficients, F-test and final response models

Yeast	Indep. variables	Response	R^2	F-test	Model	
S. cerevisiae CBS 493.94	E_d, t	$\frac{N}{No}$	0.977	258.96	$\frac{N}{No} = 0.386 - 0.615 \cdot E_d - 54.42 \cdot t + 39.136 \cdot E_d \cdot t$	(6)
C.utilis MIUG 3.5	E_d, t	$\frac{N}{No}$	0.957	13.12	$\frac{N}{No} = 0.515 - 0.987 \cdot E_d - 62.36 \cdot t + 51.96 \cdot E_d \cdot t$	(7)

3.3. Response surface plots

Based on the regression analysis previously presented, the graphical perspective of the mathematical model (equation 8) was obtained in Table Curve 3D programme. This has been done by plotting the response (reduction of yeast cell population, *N*/*N*o) as function of the independent parameters (the energetic density of light and the duration of IPL treatment) for *S. cerevisiae* CBS 493.94 (Fig. 2) and *C.utilis* MIUG 3.5 (Fig. 3).

The surfaces obtained follow the reduction of yeast cells, decreasing continuously until reach the lowest level which is the total destruction of viable cells.

In each of the plots, this level is represented by *N*/*N*o = 0 and certain values of energetic density of light and duration of IPL treatment. It is obvious that total reduction of yeast cells can be obtained through several equivalent treatments such as 0.36 J/cm^2/pulse and ten pulses or 0.72 J/cm^2/pulse and six pulses.

Besides the response surface plots as function of independent parameters, RSM study allows to determine the optimum points of the process. In this study, any point situated at the base of the plot, having *N*/*N*o = 0 and certain values for energetic density and IPL treatment duration, represents an optimum point. Therefore, it is possible to talk about an optimal area of the response surface.

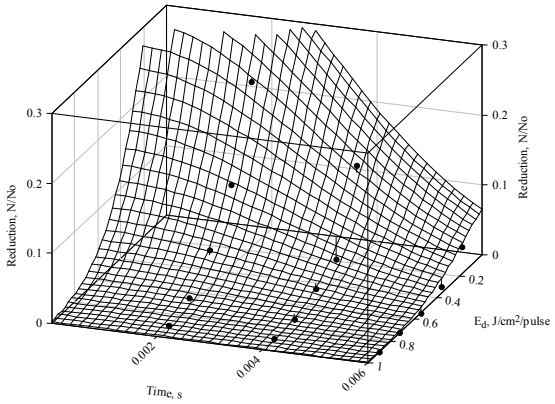

Fig. 2. The response surface as a function of the independent parameters for *S. cerevisiae* CBS 493.94 yeast cells

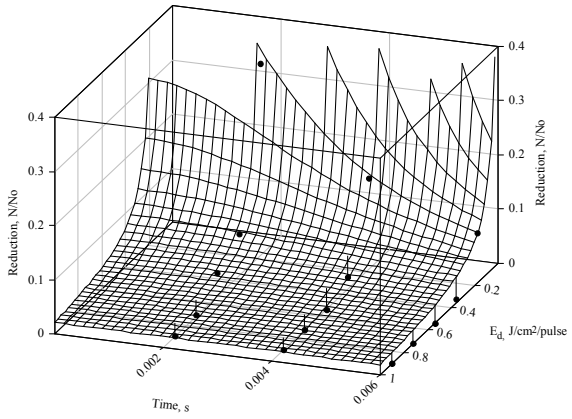

Fig. 2. The response surface as a function of the independent parameters for *C.utilis* MIUG 3.5 yeast cells

4. Conclusion

This study demonstrates that IPL treatment holds promise for the reduction of yeast cells in suspension. In addition to that, the RSM study allows to define the relationship between the response, *N*/*N*o, and the independent variables (energetic density, *Ed* and duration of treatment, *t*). It generates a mathematical model derived from the quadratic equation through regression analysis and excluding the irrelevant coefficients. The model obtained shows a direct dependency of the yeast cells reduction by independent variables presented in the first order terms. The second order terms are missing in the model which indicates the simplicity of the destruction process. Besides that, the presence in the model of the last term which contains the product of independent variables is present reflects certain binary interactions of the parameters of ILP process, the energetic density and the process duration.

The response (reduction of yeast cell population, *N*/*N*o) plotted as a function of independent variables (the energetic density of light and the duration of IPL treatment) allows obtaining the response surface for each of yeast cells population. The response surface decreases when higher energetic densities or IPL treatment times are applied and has an optimal area when reduction of yeast cell population, *N*/*N*o, is equal with zero.

As expected, ILP treatment has a powerful effect on yeast cells destruction. Shorter treatment durations are necessary to destroy the same number of viable cells when higher energy densities are applied.

Acknowledgements

The authors wish to thank FOODSEG Project (FP7 266061 contract) under the 7th RTD Framework for the support provided.

Abbreviations

AAD – absolute average deviation
CFU – colony forming units
FCD – face centred design
HHP – high hydrostatic pressure
IPL – intense pulsed light
MEA – malt extract agar
PEF – pulsed electric field
RSM – response surface methodology
RTE – ready-to-eat
UV – ultraviolet

List of symbols

a_i, a_1, a_2 – regression coefficients for linear;
a_{ii} , a_{11}, a_{22} – regression coefficients for quadratic;
a_{ij}, a_{12} – interaction coefficients;
a_o – regression coefficient for intercept;
E – light intensity, in J;
E_d – Energetic density of light, in J/cm^2;
N – number of living cells after IPL treatment, in CFU/ml;
No – number of living cells before ILP treatment, in CFU/ml;
p – number of experimental run;
R^2 – coefficient of determination;
t – time, in s;
X – coded variable;
x – natural variable;
x_1, x_2 – independent variables;
X_i, X_j – coded independent variables;
x_{max} – maximum values of natural variable;
x_{min} – minimum values of the natural variable;
y – dependent variable (response);
$y_{i,cal}$ – calculated responses;

$y_{i,exp}$ – experimental responses;
λ – wavelength of electromagnetic field, in nm;

Compliance with Ethics Requirements

Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human and/or animal subjects (if exists) respect the specific regulations and standards.

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