academicJournals

Vol. 13(45), pp. 4259-4267, 5 November, 2014
DOI: 10.5897/AJB2014.13643
Article Number: CB147EB48416
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Statistical optimization of lactic acid production by *Lactococcus lactis* strain, using the central composite experimental design

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Received 19 January, 2014; Accepted 6 October, 2014

The individual and interactive effects of a total inoculums size (% v/v), fermentation temperature and skim milk dry matter added (% w/v) on the lactic acid production by *Lactococcus lactis* LCL strain were studied by quadratic response surface methodology. The central composite design (CCD) was employed to determine maximum lactic acid production at optimum values for process variables and a satisfactory fit model was realized. The mathematical relationship of the lactic acid production on the three significant independent variables can be approximated by a nonlinear polynomial model. Predicted values were found to be in a good agreement with experimental values (R^2 of 96.7% and R^2*(adj)* of 92.1% for response *Y*). The result of optimization predicted by the model has shown that the maximal result for lactic acid production revolved around 92°D at the optimal condition with 2% of inoculums size, temperature at 30°C and skim milk dry matter added at a central point of 2% (w/v).

Key words: Central composite design, *Lactococcus lactis*, lactic acid production, inoculum size, temperature, skim milk dry matter.

INTRODUCTION

The manufacture of fermented foods has a long tradition. At first, there was a purely empirical principle without the connection between metabolic activity of microorganisms (so-called "house flora") and desired changes in the product (Geisen et al., 1992). The fermentation process was used to improve shelf-life and safety of foods enabling people in moderate and cold regions to survive winter seasons and drought periods (Holzapfel, 1997). Spontaneous fermentation of foods is characterized by the participation of lactic acid bacteria, Gram-positive, catalase-positive cocci, yeasts and moulds (Buckenhüskes, 1993). Fermented milks are the most common products from which other products are also made (Thapa, 2000). Starter culture organisms used in

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Abbreviations: RSM, Response surface method; **CCD,** central composite design; **BBD,** box-behnken design; **IDF,** international dairy federation; **DOE,** design of experiment.

fermentations belongs to a family of bacteria collectively known as the lactic acid bacteria (LAB). Fermented milks are products prepared by controlled fermentation of milk to produce acidity and flavor to desired level. Modern starter cultures are selected either as single or multiple strains, specifically due to their adaptation to the substrate or raw material (Holzapfel, 2002). The inoculation of milks with a starter culture composed of selected lactic acid bacteria that improves quality, safety, properties standardization, including flavor and color, and shortening in the ripening time (Leroy et al., 2006; Rantsiou et al., 2005). On a technological standpoint, these bacteria are invited to play the technological part to which they were selected, namely; the production of lactic acid, aromatic compounds, and production of CO_2, bacteriocins, resistance to phages, proteolytic activity and autolytic potential (Gibbs, 1987; Frey, 1993; Huang et al., 1994; Albenzino et al., 2001; Beresford et al., 2001; Hassaïne et al., 2007).

One of the most sought technological properties in lactic acid bacteria, is undoubtedly the production of lactic acid, because this activity is essential in the early stages of product processing and thereafter is mainly responsible for microbial stability of the final product through the pH decrease (Drosinos et al., 2007). This acid is widely employed as bacterial biopreservative in foods (Ray and Sandine, 1992) and recently, as monomer for the plastic polymer synthesis, solvents and oxygenated chemicals (Datta et al., 1982; Datta and Henry, 2006).

These last years, the lactic acid production has received increased attention sanctioned by a considerable number of publications (Yu et al., 1997; Lei et al., 2008; Plessas et al., 2008; Yu et al., 2008 ; Adesokan et al., 2009; Cristian et al., 2009; de Lima et al., 2009 ; Yao et al., 2009; Cristian et al., 2010; de Lima et al., 2010; Abdel-Rahman et al., 2011; Coelho et al., 2011; Kostov et al., 2011; Leite et al., 2012; Dwivedi et al., 2012; Tanyildizi et al., 2012; Ghaffar et al., 2014). In these studies, wide varieties of products and raw materials from the food and/or agriculture industries have been employed for microorganism growth due to their considerable availability and low cost. Examples include cheese whey, corn steep liquor, corn syrup, distillery yeast and molasses (Lei et al., 2008; Mussatto et al., 2008; Yu et al., 2008; Ben-Kun et al., 2009; Yao et al., 2009; Abdel-Rahman et al., 2011; Gowdhaman et al., 2012).

Biotechnological processes for the production of lactic acid usually include lactic acid fermentation. There have been numerous investigations on the development of biotechnological processes for lactic acid production, with the ultimate objectives to enable the process to be more efficient and economical by using strategies for optimization, based mainly on the modeling methodology (Yu et al., 2008; Cristian et al., 2009; Yao et al., 2009; de Lima et al., 2009; Cristian et al., 2010; de Lima et al., 2010; Muthuvelayudham and Viruthagiri, 2010; Coelho et al., 2011; Kostov et al., 2011, Dwivedi et al., 2012; Gowdhaman et al., 2012; Tanyildizi et al., 2012; Saravanan et al 2012; Leite et al., 2012). On the other hand, an indispensable tool for the optimization, control, design and analysis of the combined production of lactic acid to industrial scale derived the development of mathematical robust models, formulated with parameters of clear biological significance and statistically consistent which can be easily implemented in miscellaneous applications. Compared with conventional methods, the response surface method, commonly called a "RSM", is a time and labor saving method, which also reveals the interaction between the components of a reacted medium and seek the physical and chemical optimum levels (Ghadge and Raheman, 2006; Tang et al., 2004). RSM mainly consisted of the central composite design, the box-behnken design, the one factor design, the D-optimal design, the user-defined design, and the historical data design. The central composite design (CCD) and the box-behnken design (BBD) were the most used response surface design methods, which had 5 and 3 levels, respectively for one numeric factor. Central composite design (CCD) (Box and Wilson, 1951) is an experimental strategy for seeking the optimum conditions for a multivariable system, and it is an efficient technique for optimization.

The method was used to evaluate the coefficients in a quadratic mathematical model. The main purpose of this study was to perform the CCD in order to investigate the effect of total inoculums size (% v/v), fermentation temperature and skimmed milk dry matter added (% w/v) on the lactic acid production and for optimization of these parameters.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Lactococcus lactis LCL strain, used throughout this work belonged to the collection of "Laboratoire de Biologie des Microorganismes et Biotechnologie" of Oran University (Algeria). This strain was maintained on M17 broth or 10% (w/v) skim milk and deep-frozen at -20°C. As required, this culture was thawed and reactivated by two transfers in 10% (w/v) skim milk (30°C, 24 h).

Acidification activity

The lactic acid concentration was measured according to the International Dairy Federation (IDF, 1995). After subculturing in M17 Broth and 10% (w/v) skim milk in succession at 30°C for 24 h, the microbial culture was inoculated in reconstituted sterile non-fat dry milk 10% (w/v) at a level described in CCD tables (Tables 1 and 2). Titrable acidity was determined after 7 h of incubation; it is followed by measuring the Dornic acidity that expressed the acidity developed in the medium by transformation of lactose into lactic acid. Experiments were carried out in triplicate.

Design of experiment (DOE)

Experiment was conducted at "Laboratoire de Biologie des

Table 1. Experimental factors and levels investigated on the lactic acid production.

Variable	Symbol	Range and level				
		Lowest	Low	Center	High	Highest
		-α	-1	0	+1	+α
Total inoculums size (% v/v)(*I*)	x_1	0.32	1	2	3	3.68
Fermentation temperature (°C) (*T*)	x_2	21.6	25	30	35	38.4
Skim milk dry matter added (% w/v)(*DM*)	x_3	0.32	1	2	3	3.68

Table 2. Central composite design (CCD) for optimization of three variables (each on five levels) in mathematically predicted and experimental values for the production of lactic acid by *Lactococcus lactis* LCL strain.

Test number	Coded level of variables			Actual level of variables			Lactic acid production (D°)	
	x_1	x_2	x_3	Inoculum size (I %)	Fermentation temperature (T°C)	Skim milk dry matter added (DM %)	Observed values	Predicted values
1	-1	-1	-1	1	25	1	20	25.22
2	+1	-1	-1	3	25	1	48	49.60
3	-1	+1	-1	1	35	1	66	62.19
4	+1	+1	-1	3	35	1	80	79.07
5	-1	-1	+1	1	25	3	35	38.71
6	+1	-1	+1	3	25	3	52	58.60
7	-1	+1	+1	1	35	3	53	54.19
8	+1	+1	+1	3	35	3	69	66.57
9	-α	0	0	0.32	30	2	40	37.57
10	+α	0	0	3.68	30	2	70	68.45
11	0	-α	0	2	21.6	2	36	27.14
12	0	+α	0	2	38.4	2	60	64.89
13	0	0	-α	2	30	0.32	70	70.10
14	0	0	+α	2	30	3.68	75	70.93
15	0	0	0	2	30	2	93	92.23
16	0	0	0	2	30	2	92	92.23
17	0	0	0	2	30	2	91	92.23

Microorganismes et Biotechnologie" and was designed by central composite design (CCD). It was chosen to show the statistical significance of the effects of total inoculums size (% v/v), fermentation temperature and skimmed milk dry matter added (% w/v) on the lactic acid production by *L. lactis* LCL strain. The experiments were designed by using the STATISTICA v.7.0 software package (StatSoft, USA).

CCD allows estimating the second-degree polynomial of the relationships between the factors and the dependent variable and gives information about interaction between variables (factors). The lowest and the highest levels of variables are shown in Table 1. A 2^3 factorial central composite design with eight star points, and three replicates at the center points leading to 17 runs were employed for the optimization of the culture conditions. The variables were coded according to the following equation (Equation 1).

$$x_i = (X_1 - X_0) / \Delta X \qquad i = 1, 2, \ldots\ldots, k \qquad (1)$$

Where, x_i is the dimensionless value of a variable, X_1 the real value of a variable, X_0 the value of X_1 at the center point, and ΔX the step change. The central composite design including the factors, their levels and the result from each test, is shown in Table 2. The second-order polynomial equation, which includes all interaction terms were used to calculate the predicted response (Equation 2).

$$\gamma_i = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_{ii}^2 + \sum_{ij=1}^{k} \beta_{ij} x_i x_j \qquad (2)$$

Where, γ_i is the predicted response, x_i and x_j the input variables, the intercept term, β_i the linear effects, β_{ii} the squared effects and β_{ij} the interaction term. The design expert software has been used for regression and graphical analysis of the obtained data. The optimum levels of total inoculums size (% v/v), fermentation temperature and skimmed milk dry matter added (% w/v) were obtained by solving the regression equation and also analysis of the response surface contour plots.

Statistical data analysis

STATISTICA v.7.0 software package (StatSoft, USA) was used for the experimental design matrix, data analysis and quadratic model

Table 3. Model coefficient estimated by linear regression.

Factor	Coefficient	standard error	Computed t-value	P-value	Statistical significance of coefficient
Intercept	92.2345	3.368031	27.38528	0.00000	*
x_1	18.3806	3.165163	5.80717	0.000659	*
x_1^2	-27.7883	3.486988	-7.96913	0.000093	*
x_2	22.4730	3.165163	7.10012	0.000194	*
x_2^2	-32.7486	3.486988	-9.39165	0.000032	*
x_3	0.4984	3.165163	0.15745	0.879335	
x_3^2	-15.3875	3.486988	-4.41282	0.003108	*
$x_1\ x_2$	-3.7500	4.133659	-0.90719	0.394464	
$x_1\ x_3$	-2.2500	4.133659	-0.54431	0.603125	
$x_2\ x_3$	-10.7500	4.133659	-2.60060	0.035394	*

$\mathbf{x_1}$: Inoculum size (I %); $\mathbf{x_2}$: fermentation temperature (T°C); $\mathbf{x_3}$: Skim milk dry matter added (DM%)

Table 4. Analysis of variance (ANOVA) for the second-order polynomial model.

Source	Sum of squares	Degrees of freedom	Mean of square	F-test	P-value
Model	7181.83	9	797.98	23.35	0.0005*
Residual error	239.22	7	34.17		
Total	7421.05	16			

*Statistical significance; R^2=0.967; R^2_{adj} =0.921; R=0.983 and R_{adj}=0.959.

building. Response surface and contour plots were generated to understand the interaction of different variables. The central composite design including the factors, their levels, and the result from each test is shown in Table 2.

RESULTS AND DISCUSSION

The central composite design matrix of the studied variables: inoculum size (x_1), temperature (x_2) and skim milk dry matter added (x_3) using the isolated *L. lactis* LCL. The highest lactic acid production achieved in the verification experiment was 93.00 °D (as seen in run 15). The application of multiple regression analysis methods yielded the following regression (Equation 3) for the experimental data demonstrated that lactic acid production was an empirical function of test variables in coded units.

$$Y = 92.2345 + 18.3806\ x_1 + 22.4730\ x_2 + 0.4984\ x_3 - 27.7883\ x_1^2 - 32.7486\ x_2^2 - 15.3875\ x_3^2 - 3.7500\ x_1\ x_2 - 2.2500\ x_1\ x_3 - 10.7500\ x_2\ x_3 \quad (3)$$

The quadratic model in Equation 3, with nine terms, contains three linear terms, three quadratic terms and three factorial interactions, in which Y is the predicted response, that is, lactic acid concentration and x_1, x_2 and x_3 are the coded values of the test variables inoculum size, temperature and skim milk dry matter added, respectively.

Table 3 displays the Student's *t*-distribution and the corresponding values, along with the estimated parameters. The probability (*p*) values were used as a tool to check the significance of each coefficient. A larger magnitude of the *t*-test and smaller *p*-value denote greater significance of the corresponding coefficient (Lee and Wang, 1997; 2001; Li and Lu, 2005).

The results reveal (Table 3) that the independent variables x_1 and x_2 had a strong positive linear effect on the response (P < 0.05), as an increase in its concentration led to an increased yield. The same is observed with the squared variables (x_1^2, x_2^2, x_3^2) and the interaction term $x_2\ x_3$; the negative signs revealed a reduction in lactic acid production when its concentration was increased in the system.

Among these, insignificant terms (on the basis of *P*-values greater than 0.05) are neglected, that is, the case of the independent variable x_3 was not significant within the range of this study. The Equation 3 model was modified to reduce the fitted model (Y_a) (Equation 4).

$$Y_a = 92.2345 + 18.3806\ x_1 + 22.4730\ x_2 - 27.7883\ x_1^2 - 32.7486\ x_2^2 - 15.3875\ x_3^2 - 10.7500\ x_2\ x_3 \quad (4)$$

The statistical significance of Equation 4 was checked by an *F*-test and the analysis of variance (ANOVA) for the quadratic response surface model is summarized in Table 4. The model *F*-value of 23.35 with a very low

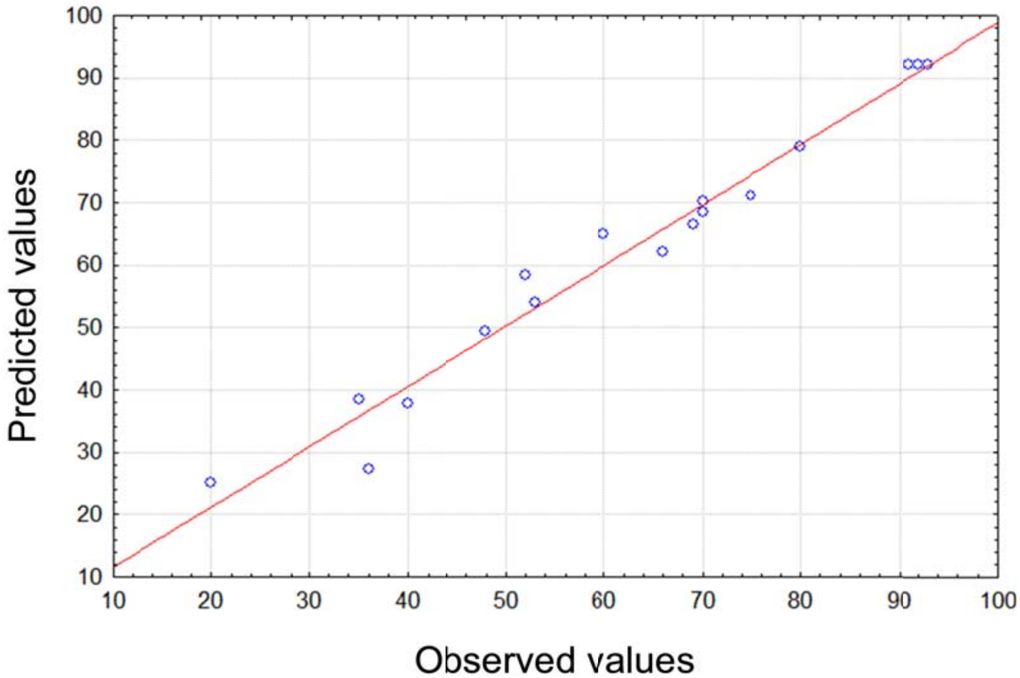

Figure 1. Relation between experimental (observed) and predicted value of lactic acid production using equation 4.

probability value (P-value = 0.0005) indicated that the model was highly significant. Experimental results and the predicted values obtained by using model (Equation 4) are shown in Figure 1. As it can be seen, the predicted values match the experimental values reasonably well with R^2 of 0.957 and adjusted R^2 of 0.921. The high R-value (0.983) demonstrates strong agreement between the experimental observations and predicted values. This correlation is also confirmed by the plot predicted versus experimental values of lactic acid production in Figure 1, as all points cluster around the diagonal line, demonstrating that no significant violations of the model were found. The goodness of the model was checked by the determination coefficient (R^2). In this case, the R^2-value (0.967) for Equation 4 indicating that 96.7% of the variability in the response could be explained by the model. Normally, a regression model with an R^2-value greater than 0.9 is considered as having a very high correlation (Rao et al., 2006). The value of the adjusted determination coefficient (adjusted R^2 = 0.0.921) was also satisfactory for confirming the good significance of the model. The high R-value (0.983) demonstrates a high degree of agreement between the experimental observations and predicted values.

The 3D response surface plot is a graphical representation of the regression equation. It is plotted to explain interaction of the variables and locate the optimal level of each variable for maximal response (Figures 2, 3 and 4). Each response surface plotted for lactic acid production represents the different combinations of two test variables at one time while maintaining the other variable at the zero level. These 3D plots and its respective contour plots provide a visual interpretation of the interaction between two factors and facilitate the determination of optimum experimental conditions.

The convex response surfaces suggest that there are well-defined optimal solutions. If the surfaces are rather symmetric and flat near the optimum, the optimized values may not vary widely from single variable conditions (Rao et al., 2006). Interactions between variables can be inferred from the shapes of the contour plots. Circular contour plots indicate that interactions between variables are negligible, as shown in Figure 2. In contrast, elliptical plots indicate interactions, as it is shown in Figures 3 and 4 (Muralidhar et al., 2003). The inoculum size and the fermentation temperature seem to be dominant variables in lactic acid production model (Figures 2 and 3). Whereas, the skim milk dry matter added (on linear term) does not seem to have a notable effect on this production (Figures 3 and 4). The maximal lactic acid production occurred when inoculum size and temperature were in the neighborhood of 2% (v/v) and 30°C, respectively.

The area of optimum lactic acid production levels of the tested variables is located close to the central point, and they were represented in desirability charts (Figure 5) and isoresponse plot (Figure 6), constructed using response surface regression in STATISTICA software. The point of maximal lactic acid production was determined through canonical analysis of the adjusted

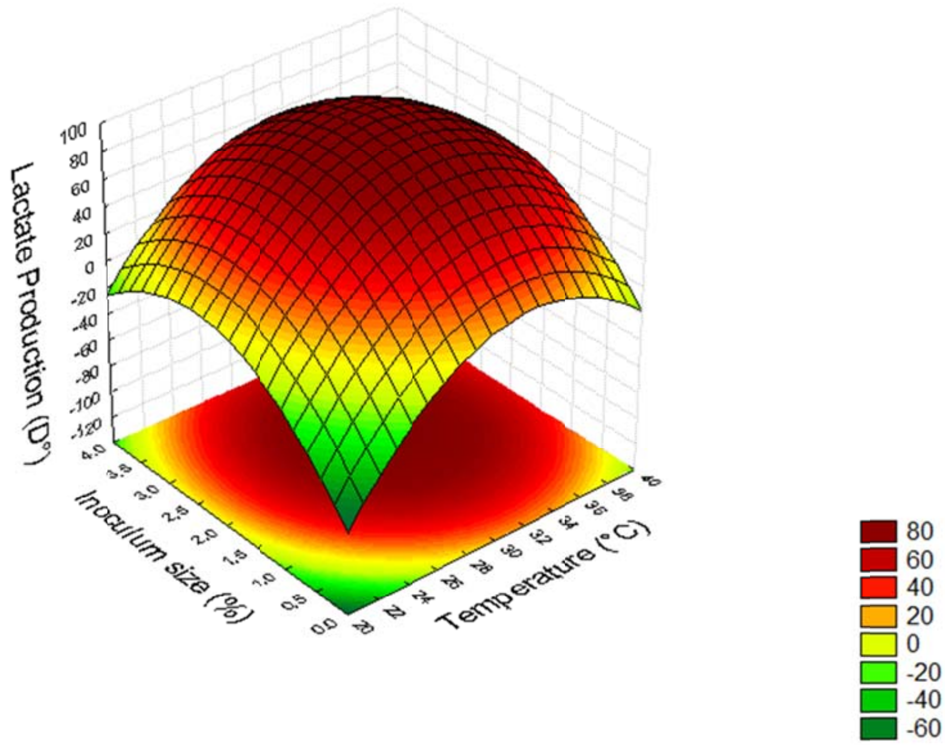

Figure 2. Response surface plot showing the effect of inoculums size and temperature on lactic acid production. The value of the variable skim milk dry matter added was fixed at the central point.

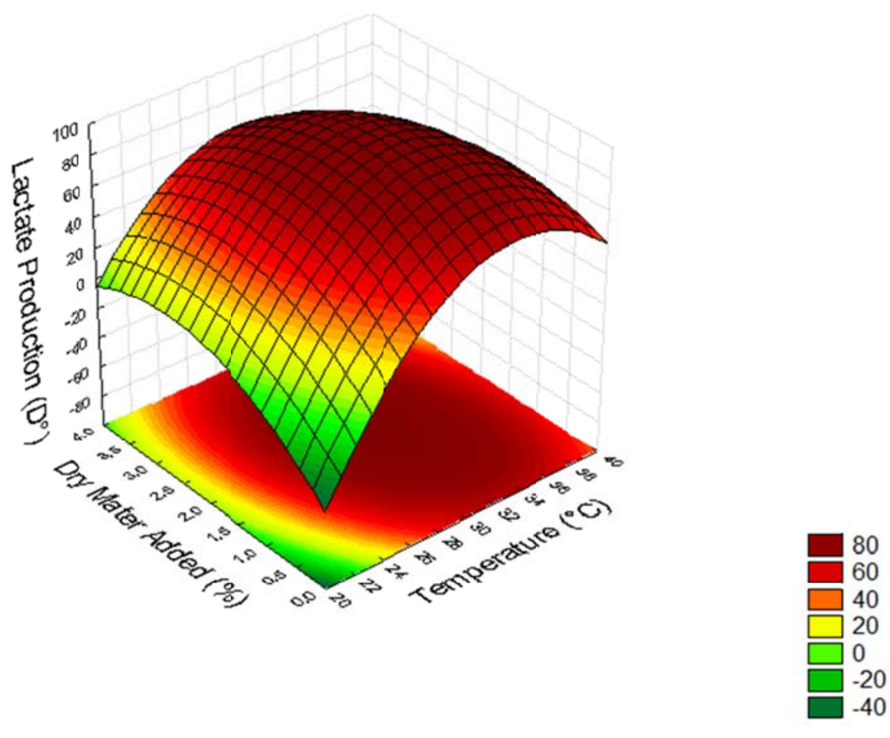

Figure 3. Response surface plot showing the effect of skim milk dry matter added and temperature on lactic acid production. The value of the variable inoculums size was fixed at the central point.

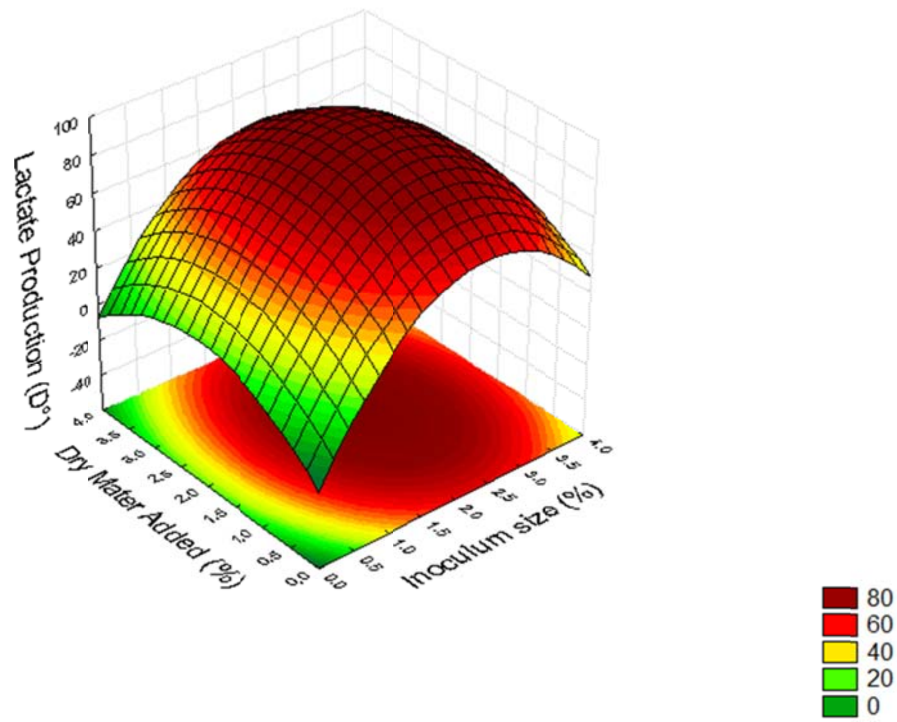

Figure 4. Response surface plot showing the effect of skim milk dry matter added and inoculums size on lactic acid production. The value of the variable temperature was fixed at the central point.

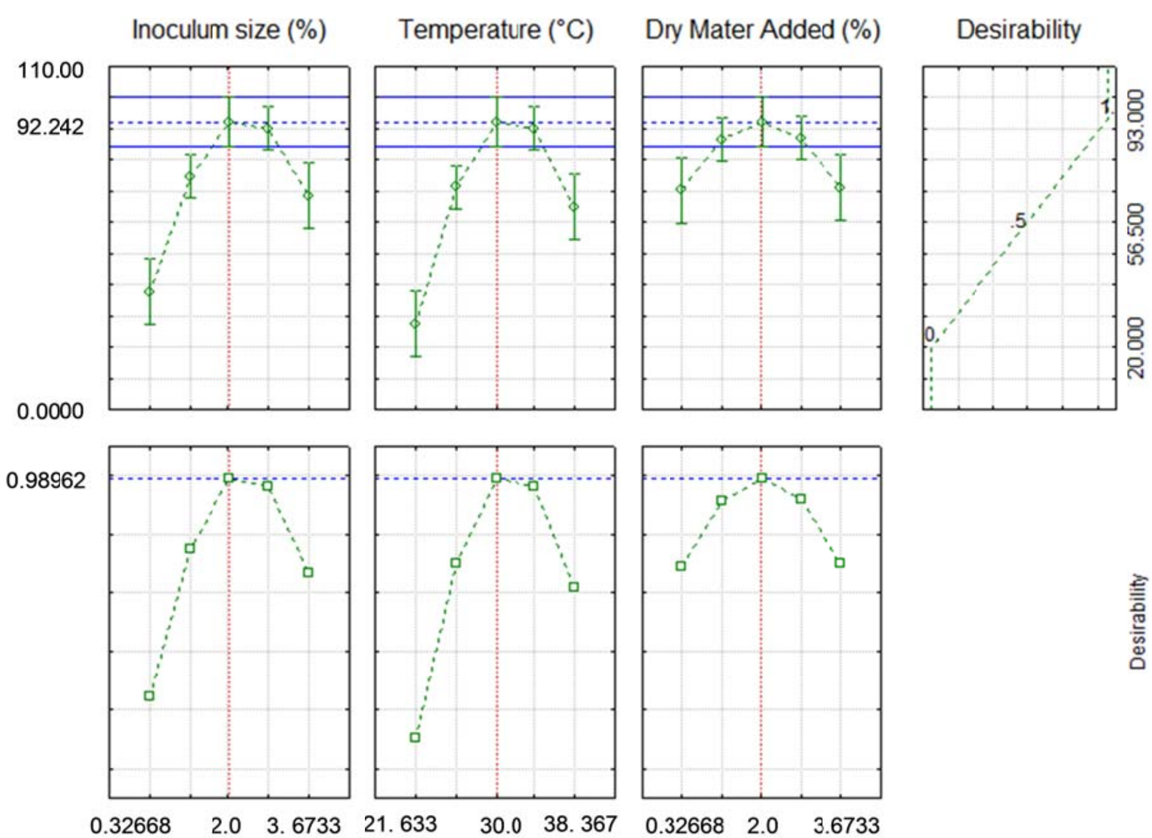

Figure 5. Desirability charts of variables for maximum response (lactic acid production).

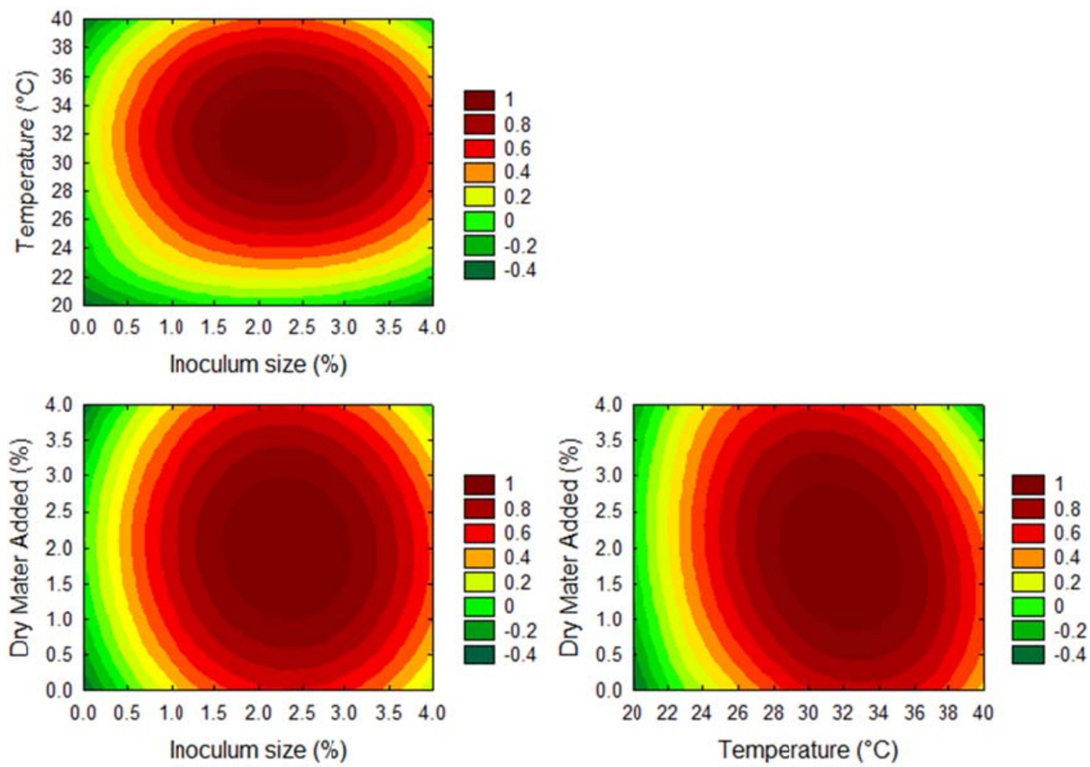

Figure 6. Desirability isoresponse plot of variables for maximum response (lactic acid production).

model. A study was carried out to identify the nature of the stationary point (maximal point or low response or still of a saddle point). These levels were as follows: inoculum size 2% (v/v), temperature 30°C and skim milk dry matter added to 2% (w/v) for 92.24 °D predicted value of lactic acid production. To confirm the adequacy of the model for predicting maximal lactic acid production, three additional experiments were also conducted at these predicted optimum levels. The mean value of lactic acid concentration obtained is 92 ± 0.5 °D, which is an excellent agreement with the predicted value.

Conclusion

It is possible to affirm that the controlled inoculums size, the temperature of fermentation and skim milk dry matter added influenced the predictive model for maximal lactic acid production by *L. lactis* LCL strain by using the central composite design method and response surface analysis. The optimization of the analyzed responses demonstrate that the best result for lactic acid production revolves around of 92 °D was obtained with 2% (v/v) of inoculums size, temperature at 30°C and skim milk dry matter added at a central point 2% (w/v).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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