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Statistical Optimization of Lactic Acid Production by the *Lactococcus lactis* DBH10 Strain in a New Culture Medium Based on Date Waste (DJM), Using Response Surface Methodology

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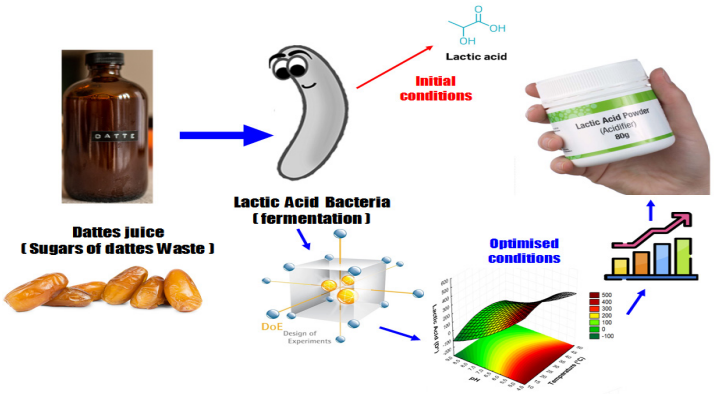
Graphical Abstract



Abstract

We have successfully applied in this work the response surface methodology (RSM) involving central composite planes (CCD) in order to study the effect of the temperature of incubation (x_1), pH (x_2) and Inoculum size (x_3) on lactic acid production by the *Lactococcus lactis* DBH10 strain. After statistical analysis, the quadratic terms of the variable (x_1^2, x_2^2, x_3^2) as well as those of the variables (x_2, x_3) in linear terms and their interactions (x_1x_2; x_1x_3) were found to have significant effects on the response of interest. The analysis of the second order polynomial regression model obtained by multiple regression analysis, allowed us to reach a maximum lactic acid concentration of 486 ± 2.4 °D. The mathematical relationship of lactic acid production by *Lactococcus lactis* DBH10 strain related to the three significant independent variables was performed by a satisfactory fitting model. The predicted values were found to be in good agreement with the experimental values, displaying a coefficient of determination of R2 = 0.989, demonstrating good significance of the proposed model. The adjustment of the levels of the factors predicted by the model resulted in a maximum lactic acid concentration, when the optimal combination of inculum size, temperature, and pH, are respectively at 2.8% (v/v), 21.5 °C and 4.8. The Validation of the predicted model was carried out by performing three more experiments at the optimal levels of these variables, thus confirming its robustness.

Keywords: Dates juice, Lactic acid production; *Lactococcus lactis*; Response Surface Methodology.

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Introduction

The date is a fruit marketed worldwide as a valuable confectionery, and as a fresh fruit. The energy recovery of its by-products is part of an economic and environmental approach. The whole date fruit is traditionally used to prepare a wide range of date products such as date paste, date syrup, date honey, spread, date jam, date vinegar and sugar [1,2].

In Algeria, date production is considerable, and constitutes an important export product. However, a massive amount of the harvest is wasted, mainly due to over ripeness of product, poor handling during processing, disruption of the storage, packaging and marketing process. [3], reported that 60 000 tons of dates are lost every year in Algeria, representing a real economic loss, as they are rich in noble components, including a large part of sugars in addition to dietary fiber, phenolic compounds, minerals, vitamins and antioxidant compounds [4-6].

Due to their high sugar content and relatively long shelf life, dates offer many technological possibilities; depending on the processing they undergo (Rambabu et al., 2020). Indeed, dates can be used as raw material for the production of various metabolites [7-9]). This richness of dates is a renewable resource that should be used efficiently in biotechnology. Indeed, the use of microorganisms is essential to achieve these specific objectives. Among these, we can mention the possibilities of bioconversions and transformations of this source into lactic acid, which seem to be a promising and beneficial alternative, given the growing interest that this metabolite has shown in recent years [9].

The lactic acid production by microbial fermentation has significantly expanded in the last decade [10,11]. Unfortunately, this kind of process requires high cost substrates (glucose, lactose,...), which make its use impractical. However, starchy materials, such as whey, starch hydrolysates and wheat can serve as a carbon source and are potential raw materials for lactic acid production [9,12,13]. In this kind of process, the culture medium is a crucial element for the growth of these bacteria and consequently the production of biomass and/or metabolites of interest, which absolutely requires the grouping of optimal conditions of nutritional qualities and technological parameters of the raw materials committed at low cost and market availability [14].

Bio-waste is considered a nutrient-rich niche, which in most cases is sufficient to meet fermentation performance [15]. In this regard, several mathematical models have been developed to optimize the fermentation process in order to obtain high yields of high value-added bioproducts [9,16].

In the same direction, an optimization of the essential parameters for the production of these metabolites of interest, namely lactic acid in our study, are of considerable scope for subsequent exploitation for practical purposes. These parameters can be statistically optimized using the responses surfaces methodology (RSM), leading to an increase effectively the conversion rate of the product and the productivity of the process, which leads to a reduction in costs and time.

In this order of ideas, the objective of the present study finds its way, whose main purpose is the valorization of date's waste, through its juice, which largely composes a new culture medium (DJM), very interesting on many levels, used for the development of *Lactococcus lactis* strain LCL and the maximum production of lactic acid. This study is essentially based on the use of statistical optimization methods to produce and predict a maximum level (concentration) of lactic acid in this medium (DJM) by this strain at different conditions considered.

Materials and methods

Bacterial Strain and Culture Conditions. The bacterial strain *Lactococcus lactis* DBH10, used throughout this investigation was obtained from the culture collection of "Laboratoire de Biologie des Microorganismes et Biotechnologie" of Oran 1 University (Algeria). This strain was routinely cultivated at 30 °C in MRS or 10% (w/v) skim milk, and stored at -20 °C in skim milk.

Date Juice Medium (DJM) preparation. The date's juice preparation was performed according to the method described by [17]. The substrate was obtained from ripe dates fruits with low commercial value. These fruits also include the ones considered as a waste product due to excessive sun exposure, damage caused by insects or fungi, deterioration during packaging and transport, and that have passed the normal stage of ripening (over-ripening).

Firstly, all the dates were washed, pitted and cut into small pieces, to which distilled water was added at the rate of two litters per kilogram. This mixture is brought to a water bath at 80 °C for 2 hours and thereafter, the extract obtained is freed from solid debris by filtration followed by centrifugation at 5000 rpm for 30 min to further separate the residual cellulosic debris still present in the supernatant (date extract). The obtained dates juice is subsequently sterilized at 110 °C for 10 min and stored at 4 °C until use.

The preparation of the culture medium (DJM) were carried out lastly by mixing aseptically 7% of date juice with a sterilized (120 °C for 20 min) base solution, composed of 250 mM of K_2HPO_4 / KH_2PO_4 phosphate buffer and 2.6 g /l of yeast extract.

Acidification activity. The lactic acid produced by the bacterial strain DBH10 in the date juice medium (DJM) was measured according to the International Dairy Federation [18]. After a successive subculturing in the JMD medium at 30 °C for 24 h, the microbial cultures was inoculated in the JDM at a level described in CCD tables (Table 1). The titratable acidity was determined after 18 hours of incubation by measuring the Dornic degree, corresponding to the acidity developed in the medium by transformation of lactose into lactic acid. Experiments were carried out in triplicate.

Design of Experiments (DOE) and Statistical Analysis. The response surface methodology is a set of mathematical and statistical techniques that are widely used for modeling and analysis of problems in which the response of interest is influenced by many variables and whose interest is to optimize the latter.

In this work, we have adopted a methodology based on responses surface in order to enquire the effect of interactions of three physicochemical factors (incubation temperature, culture medium pH and inoculum size) on the production of lactic acid by the DBH10 strain (*Lactococcus lactis*). For this purpose, a central composite design (CCD) matrix at 17 trials for three independent variables was used in order to assess not only the linear and quadratic effects of the three factors but also their interactions on the responses of interest in order to optimize the lactic acid production. The matrix of experiments used is shown at table 1, represents each variable studied at five levels (-α, -1, 0, +1, and +α) as described by [19].

The responses of technological interest (lactic acid concentration) relating to the 17 tests of the matrix of experiments was determined after 18 hours of incubation and analyzed using the STATISTICA v.7.0 software package (StatSoft, USA) to perform the regression and graphical analysis of the data obtained. The minimum and maximum ranges of variables were used, and the full experimental design with their coded values is shown in table 1. A first three-dimensional type of graph was generated (response surfaces) to be assess the effects of the combined interactions of factors, showing at each time Temperature-Inoculum size, Temperature-pH and Inoculum size-pH, on the response of interest.

The mathematical models for predicting linear, quadratic effects and those of interactions of factors on the responses of interest were generated, Indeed, the experimental study seeks to link a physical quantity Y (the response) and a physical quantity x (the factor). The experimental design requires the use of the linear regression technique to determine the coefficients of the model. The model developed is a polynomial function of second order comprised linear, quadratic and interaction terms whose form is as follows:

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

Where Y is the predicted response, x_i and x_j are coded independent variable, β_0 the intercept term, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient and k is number of factors.

Statistical Data Analysis. The statistical analysis was performed to assess to evaluate the significance and the relevance of model by the analysis of variance (ANOVA). The obtained equation of model was also appreciated by the Fisher's test, and the same for the proportion of variance explained by the model, it has been appreciated by examining the multiple coefficient of determination (R^2) value.

Results and Discussion

Lactic acid is one of the most important organic acids, widely used throughout the world in many industrial applications. It is also mostly used in the food, cosmetic, pharmaceutical and chemical industries [20], and has received increased attention as a precursor to polylactic acid (PLA), a biodegradable plastic used in fiber-to-packaging applications [21]. Indeed, this acid can be produced by chemical synthesis or by microbial fermentation.

This work was carried out on a new culture medium based on date juice which has been described by [17], using the *Lactococcus lactis* DBH10 strain being obviously the most productive of lactic acid. This ability to acidification, is a character often reported as being be dependent of the isolate linked to the particular abilities that the latter has to effectively use the compounds of the culture medium and thus to make it more assimilable [22]. Indeed, in this medium, the added yeast extract appears to play an important role as a nitrogen source by enhancing lactic acid production compared to other nitrogen sources that can be supplemented. In this same context, the pH factor seems to be a crucial parameter to take into account in the production of lactic acid in this medium based on date juice, presumably because of its direct impact on the proper functioning of the sugar utilization mechanism, directly affecting the enzymatic activities of these metabolic pathways [15].

The effects of three variables on the production of lactic acid by *Lactococcus lactis* DBH10 strain in JDM medium as described by [17] were studied. Table 1 presents the design matrix and the results obtained from the central composite design regarding the variables studied in coded units and in real values; the temperature of incubation (x_1), pH (x_2) and Inoculum size (x_3). The highest lactic acid production achieved in the verification experiment was 480 D° (as seen in run 11) around of 30 °C, pH at 4.8 and 2% of inoculums size.

The Statistica software was used to analyze the obtained data in order to evaluate the effects of 3 factors on the response of interest. The model coefficients were estimated by multiple linear regressions and the statistical significance of each variable and its interactions was determined by Student's *t*-distribution and *P*-values. Table 2 summarizes the result of this analysis, where the *P*-value was used as a tool for verifying the significance of each coefficient, because a larger value of the *t*-test combined with a smaller *P*-value is synonymous to a greater significance of the examined coefficient [23].

The application of multiple regression analysis method yielded that the production of lactic acid was an empirical function of tested variables in coded unit. The global mathematical equation giving the value of *Y* as response to the three studied factors was written according to the following general formula (Equation 2):

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad (2)$$

The global model proposed for the production of lactic acid by the DBH10 strain is written according to the following equation (Equation 3).

$$Y = 194.13 - 4.86\, x_1 - 125.18\, x_2 + 27.19\, x_3 - 26.79\, x_1^2 - 17.56\, x_2^2 - 45.05\, x_3^2 + 14.12\, x_1 x_2 - 5.62\, x_1 x_3 - 26.62\, x_2 x_3 \quad (3)$$

The quadratic model in equation 3, is composed of nine terms, whose three linear terms, three quadratic terms and three factorial interactions, in which *Y* is the predicted response, i.e., lactic acid concentration and x_1, x_2 and x_3 are the coded values of the variables; temperature, pH and inoculum size respectively.

Table 1. Box-Wilson Central Composite Design (CCD) matrix with three variables used for the optimization of the production of lactic acid by Lactococcus lactis DBH10 strain in JDM medium.

Runs	Coded level of variables			Actual level of variables			Lactic Acid Production (D°)	
	X1	X2	X3	Temperature (°C)	pH	Inoculum size (I %)	Observed Values	Predicted Values
1	-1	-1	-1	20	5.6	1	206	225.10
2	+1	-1	-1	40	5.6	1	186	198.27
3	-1	+1	-1	20	8	1	10	0.42
4	+1	+1	-1	40	8	1	19	30.10
5	-1	-1	+1	20	5.6	3	340	341.98
6	+1	-1	+1	40	5.6	3	270	292.66
7	-1	+1	+1	20	8	3	10	10.80
8	+1	+1	+1	40	8	3	24	17.98
9	-α	0	0	13	6.8	2	126	124.97
10	+α	0	0	47	6.8	2	126	108.47
11	0	-α	0	30	4.8	2	480	452.95
12	0	+α	0	30	8.8	2	25	33.49
13	0	0	-α	30	6.8	0.35	38	24.72
14	0	0	+α	30	6.8	3.7	118	112.72
15	0	0	0	30	6.8	2	189	194.09
16	0	0	0	30	6.8	2	188	194.09
17	0	0	0	30	6.8	2	202	194.09

α=1.68

Table 2. Model coefficient estimated by multiplies linear regression medium.

Factor	Coefficient	*t*-test	*p*-value
Intercept	194.13	22.3218	0.0000*
(x_1) Temperature (L)	-4.86	-0.3761	0.7179
(x_1^2)Temperature (Q)	-26.79	-3.1288	0.0166*
(x_2) pH (L)	-125.18	5.5407	0.0008*
(x_2^2) pH (Q)	17.56	-4.0187	0.0050*
(x_3) Inoculum size (L)	27.19	6.5053	0.0003*
(x_3^2) Inoculum size (Q)	-45.05	-8.8847	0.0000*
(x_1) Temperature (L) by (x_2) pH (L)	14.12	0.9894	0.3553
(x_1) Temperature (L) by (x_3) Inoculum size (L)	-5.62	-0.1159	0.9109
(x_2) pH (L) by (x_3) Inoculum size (L)	-26.62	-0.8918	0.4020

**: Statistical significance.*

Table 3. Analyze of variance (ANOVA) for the second-order polynomial model.

Source	Sum of Squares	Degrees of freedom	Mean of Square	*F*-test	*P*-value
Model	269353.47	9	29928.16	76.83	3.64 E-06*
Residual Error	2726.64	7	386.52		
Total	272080.12	16			

** Statistical significance; R2 = 0.98; adj. R2 = 0.97*

The obtained results reveal that the independent variables x_2 (pH) and x_3 (Inoculum Size) had a strong linear effect on the response ($P < 0.05$). The same is observed with the squared variables (x_1^2, x_2^2, x_3^2) and the interactions terms (x_1x_2; x_1x_3). In the equation, the negative sign is synonymous that a reduction of the lactic acid production in the system when the magnitude of this factor was increased. This result confirms the involvement of these three factors in the establishment of definitive mathematical models and determines the necessity and importance of adjustments at the biological level of these factors in such a process. At the end of this analysis, the statistically insignificant terms of equation (based on *P*-values greater than 0.05) are neglected and therefore they are omitted from the final equation. The Equation (3) of model is modified and reduced to the fitted model (*Ya*) by retaining only the terms having a statistically significant influence on *Y*, and the Equation (3) is rewritten as follows (Equation 4).

$$Y = 38{,}9032 + 4{,}7568\, x_2 + 5{,}3948 x_3 - 2{,}8060\, x_1^2 - 4{,}1994\, x_2^2 - 8{,}0748\, x_3^2 \qquad \text{Eq. (4)}$$

The quadratic response surface model (Equation 4) was been examined using the *F*-test and the analysis of variance (ANOVA) displaying an *F*-value of 76.83, combined with a value a very low probability value (*P*-value = 3.64 E-06), thus revealing that the proposed model is highly significant (Tab. 3). This analysis confirmed and consolidated that the proposed model supports almost all the hypotheses formulated from the measured responses.

The correlation analysis of the data obtained experimentally compared with those predicted by the model (equation 4) is shown in Table. 3, where we find that the predicted values match quite well the experimental values, displaying an R^2 of 0.989 and an adjusted R^2 of 0.976. This correlation is also confirmed by the plot of predicted versus experimental values of lactic acid production as shown in Figure (1), where the majority of points cluster around the diagonal line, which is proven that no significant violations of the model were found. The very close alignment of the points around the straight line means a strong adequacy between the experimental data and those predicted, thus reflecting the good robustness of the proposed model by displaying a high coefficient of determination.

The robustness and relevance of the model was checked by the determination coefficient (R^2) of model equation (Eq. 4). In the present study, it corresponds at the R^2-value of 0.989, indicating that the proposed model could explain 98% of the variability of the response. It is well established that a regression model with an R^2-value greater than 0.9 is considered as having a very high correlation and quite robust [24]. The value of the adjusted determination coefficient (adjusted R^2 = 0.976) was also satisfactory for confirming the good significance of the model.

The 3D response surface plot is a graphical representation of the regression equation. It is plotted to provide a visual interpretation of the interaction between two factors, facilitate the determination of optimum experimental conditions and locate the optimal level of each variable for maximal response (Fig. 2). 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.

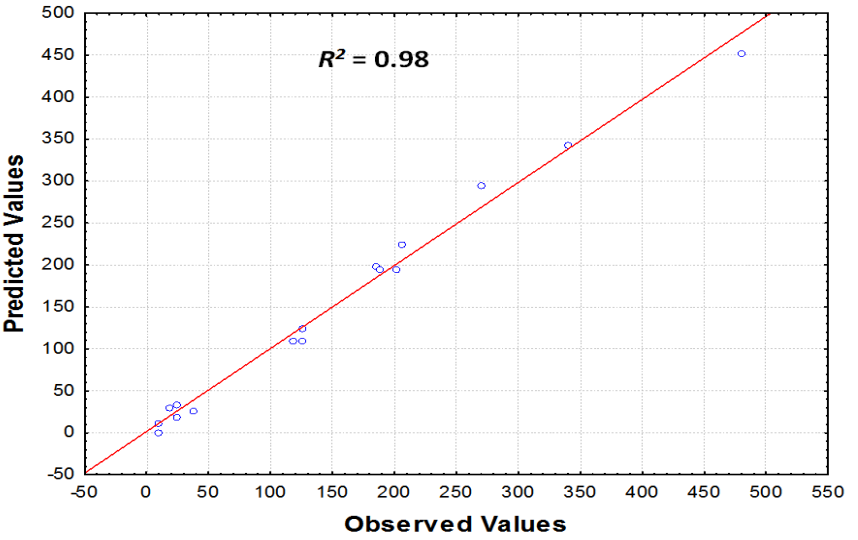

Figure 1. Relation between experimental (observed) and predicted value of the lactic acid production by *Lactococcus lactis* DBH10 strain in DJM medium.

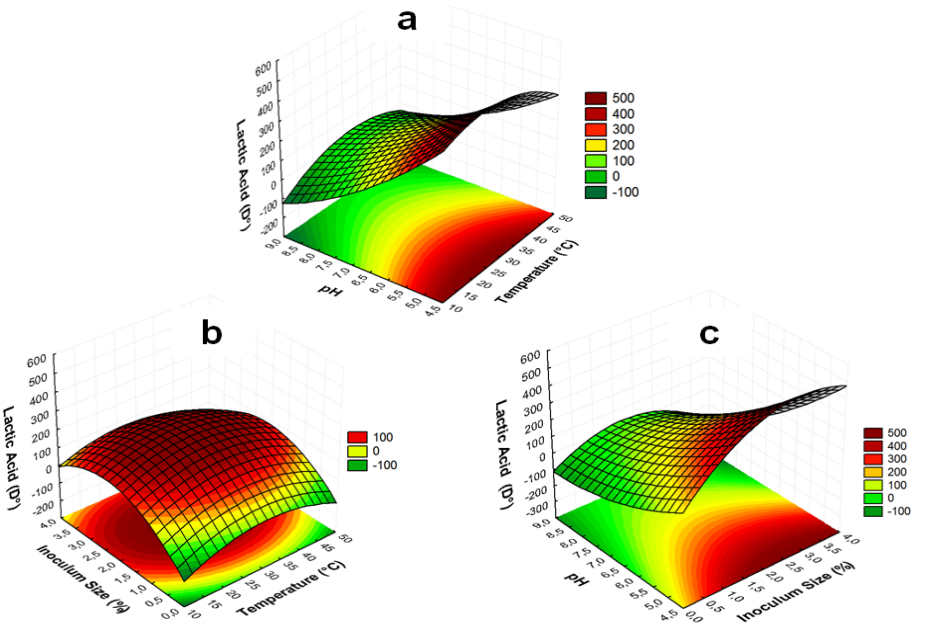

Figure 2. Response surface plot showing the effect of Temperature, pH and Inoculum Size on the lactic acid production by *Lactococcus lactis* DBH10 strain in DJM medium.

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 [24]. Interactions between variables can be inferred from the shapes of the contour plots.

On examination of these curves, it appears clearly that inoculum size and pH are variables that exert a dominant effect on lactic acid production (Fig. 2a; Fig. 2b and Fig. 2c), which is the opposite for incubation temperature (in linear terms) which does not seem to have a significant effect (Fig. 2 a; Fig. 2b). Indeed, lactic acid production is obviously maximal when inoculum size and pH are close to the values of 2 to 3% (v/v) and 4.5 to 5, respectively.

The bell shape of the curve is mathematically related to statistically significant quadratic terms of temperature and inoculum size effects. In fact, incubation temperature was shown to have a positive impact on the acidifying power of strain DBH10. This can be explained by an activation of sugar metabolism pathways and transport systems by stimulating a set of enzymes involved in the fermentation and bioconversion of sugars to lactic acid [25].

The area where lactic acid production was maximal, corresponding to optimal coordinates of the variables tested, was easily deduced graphically using the desirability function in the STATISTICA software (Fig. 3). This function is used to define the operating parameters that can generate the specified performance level of one or more responses [16]. The point of maximal lactic acid production was determined through canonical analysis of the adjusted model. In this study, we considered outcomes between 0 (undesirable) and 1 (highly desirable). These levels are as follows, where the inoculum size is 2.8% (v/v), the temperature of incubation is 21.5 °C and a pH of 4.8 for a predictive lactic acid production of 488.69 °D.

In view of validation of the proposed model and to confirm the adequacy of the factor levels obtained in this study in order to a maximum production of lactic acid, three additional experiments were also carried out at these predicted optimum levels. The mean value of lactic acid concentration obtained was of 486 ± 2.4 °D, which is in perfect agreement with the predicted value given previously.

On the basis of the results we obtained, especially those relating to the content of lactic acid produced in this new culture medium based on date juice and in these conditions, it seems to be of a high level and very interesting in comparison with many studies previously conducted in this direction, including that in milk by [26], displaying levels of concentration 5 times lower than those obtained in DJM medium, also using a *Lactococcus lactis* LCL strain.

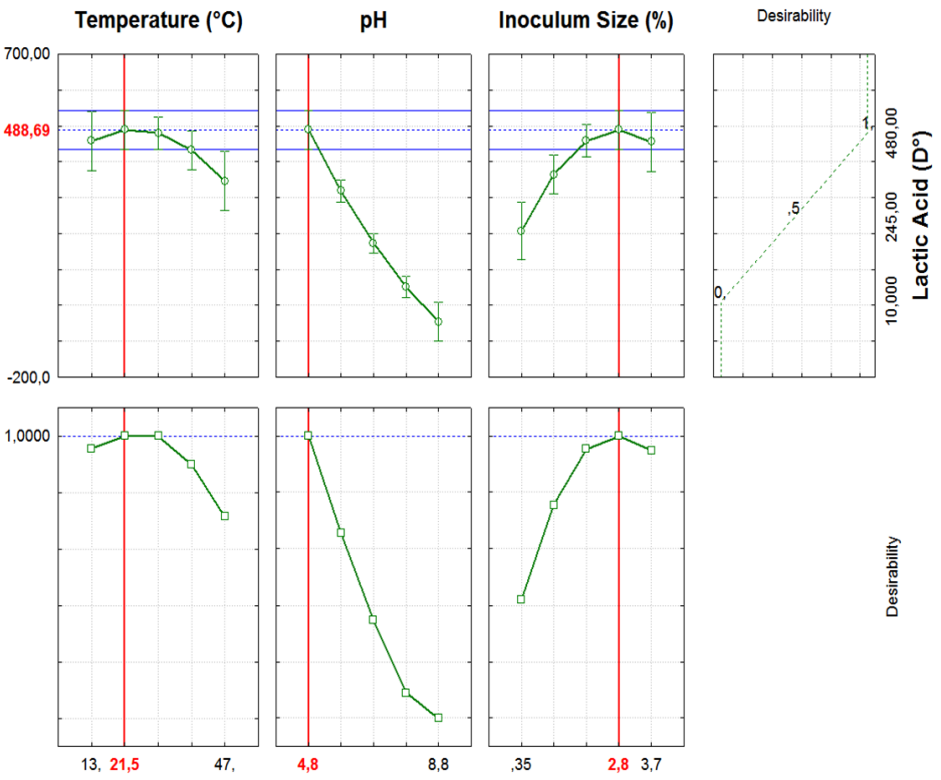

Figure 3. Desirability charts of variables for maximum response (Lactic acid production) by *Lactococcus lactis* DBH10 strain in DJM medium.

In this same sense and in the context of our work, another medium based on date juice was used for the production of lactic acid by *Lactococcus*, conducted by [27] led a concentration of 300 °D, which seems to be promising, but does not exceed the level of concentration that we have obtained in the present work which testifies to the interest of using date juice as a base for the production of lactic acid. Another interesting lactate level of 370 °D was obtained at an optimal pH of 5.5 in another study conducted by [29], or even by another study conducted by [28] leading to a concentration of 248 °D conducted at an adjusted pH of 7.

In the context of this study of optimization, the effect of temperature was examined in the range of 13 °C to 47 °C, where the maximum concentration of lactic acid was obtained at the temperature of 21.5 °C. These results greatly exceed those described by [28] who obtained a concentration of 244 °D at a temperature of 30 °C. According to [30]; the optimum temperatures for the production and maximum yield of lactic acid, were 20 and 35 °C, respectively. These studies demonstrated that among others, the pH and temperature could have a significant effect on cellular metabolism and thus lactate production, hence the importance to define and adjust them.

The optimization strategy through the use of experimental design implemented in this study using this new culture medium based on date juice, allowed to effectively improve the production of lactic acid at a concentration considered among the most interesting described in the scientific literature, displaying a concentration of 486 °D, in comparison with the highest level of 462 °D described by [31] obtained in a similar medium, based on date juice and this in comparison with the M17 medium (369 °D) under the same conditions.

Conclusion

This work has allowed refined the studied factors tuning levels and affirmed that inoculum size, temperature of fermentation, and pH, effectively influenced the predictive model for maximum lactic acid production by the *Lactococcus lactis* DBH10 strain, using the central composite design and response surface methodology. This approach of optimize lactic acid production at a maximum concentration around 486°D in the DJM medium was successfully achieved using an inoculum size of 2.8% (v/v), a temperature of 21.5°C and a pH of 4.8, following a predictive model showing a high level of robustness with an R^2 equal to 0.989.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Midoun Nacima contributed to this work both editorially and experimentally, being a significant part of her thesis project. She was followed by her thesis advisor, Professor Hassaine Omar, who supervised the various experimental and modeling components of this work in addition to contributing to the writing and editing of the manuscript.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

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