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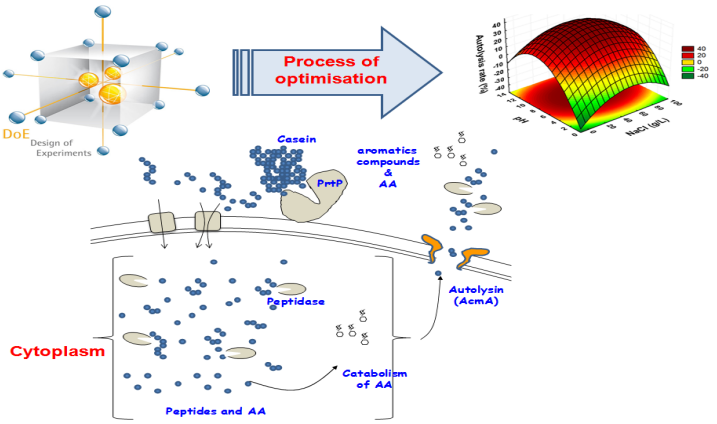
Application of Box-Wilson Central Composite Experimental Design, to Optimize the Autolysis Conditions for *Lactococcus lactis* LCL Strain; Focus on Temperature, pH and Salt Gradient

Hanane Mehiaoui[1], Salima Roudj[1], and Omar Hassaine[1*]

[1]Laboratoire de Biologie des Microorganismes et Biotechnologie, Department of Biotechnology, Faculty of Sciences of Nature and Life, Oran1 University- Ahmed Ben Bella, 31000 Oran, Algeria.

Graphical Abstract



Abstract

In this work, a Response Surface Methodology (RSM) involving Central Composite Designs (CCD) have been successfully applied to study in buffer system the effect of temperature of incubation (X1), NaCl concentration (X2) and pH (X3) on the rate of autolysis of *Lactococcus lactis* LCL strain. The mathematical relationship of the autolysis rate related to the three significant independent variables was realized by a satisfactory fit model. Predicted values were found to be in a good agreement with experimental values, displaying a satisfactory coefficient of determination R2 = 0.9586, demonstrating a good significance of proposed model. The result of optimization predicted by the model show a maximal autolysis rate, when the optimum combination of temperature, pH and NaCl concentration, are respectively at 25 °C, 7 and 52 g/L. The validation of the predicted model has been conducted by executing three more experiments at the optimal levels, confirming its robustness.

Keywords: Autolysis; *Lactococcus lactis*; Response Surface Methology; NaCl; pH; Temperature.

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Introduction

Lactic acid bacteria (LAB) are widely used as mixtures of selected bacteria to produce manifold fermented dairy products. *Lactococcus lactis* is one of the lactic acid bacterial species of major economic importance in dairy fermentations and is the most important species used in cheese manufacture [1-4]. By their large array of enzymatic contents, these bacteria play important roles during cheese manufacture by acidifying milk, following the production of lactic acid from lactose, and by development of the flavor during the ripening [5-7].

Technologically and industrial standpoint, these bacteria are invited to carry out the technological performances, for which they were selected, such as; the production of lactic acid, aromatic compounds, production of CO_2, bacteriocins, resistance to phages, proteolytic activity and autolytic potential [5-13].

Studies on the autolytic properties of cheese related micro-organisms have started in the early as 1941 [14], but, it is only well after this period that real interest was directed towards the autolysis process and its possible impact on cheese ripening [15-17]. The autolysis of *Lactococcus lactis* strains used as starters in cheese manufacture is a desirable trait, playing a key role in the cheese-ripening process, seeing that most of the enzymes related with the production of flavor compounds are intracellular, this implies that *Lactococcus lactis* cell lysis must occur first, to allow interaction between bacterial enzymes and substrates present in the cheese matrix [18,19].

The releases of intracellular enzymes into the cheese curd accelerate proteolysis and hydrolysis of large and small peptides in cheese, as well as on the accelerated production of free amino acids, which are precursors of aroma compounds, while decreasing the bitter taste [20]. For this reason, the early release of intracellular enzymes by bacterial lysis should be considered as an essential event during cheese ripening by training textural changes and accelerating the development of the characteristic flavor compounds of cheese [21-24].

Rapid autolysis of lactococcal starter strains, which causes faster release of intracellular enzymes into the cheese matrix, could possibly reduce cheese-ripening time and decrease bitterness [16,25,26], because the ripening process is a relatively expensive process for cheese industry, that requires a large storage period; and therefore, a reduction of this period without destroying the quality of matured cheese seems to be an advantage economic and technological alternative [21].

Bacterial autolysis can be defined as the spontaneous disintegration of the cell wall peptidoglycan by the endogenous enzymes, called peptidoglycan hydrolases or autolysins [15,16, 22,27-29]. The cellular lysis of LAB improves the interaction between bacterial enzymes and cheese substrates [30,31], may occur as the result of age, stress, unfavorable physiological conditions or attack of lytic phages [27]. Environmental factors such as, salt concentration, pH and temperature may affect the autolysis of *Lactococcus lactis* [24]. Several authors examined the influence of different physicochemical factors on the rate of autolysis for different LAB species.

The autolysis has been induced by transferring the cells in a buffer solution in which the cells have been in nutritionally starvation condition [32]. For this purpose, various factors such as growth phase, pH, temperature, activators and inhibitors influencing cell lysis can be studied. The general conclusion indicates that the optimum temperature of autolysis varies according to the organism tested [33-38]. These studies, even though they are few, have shown that high rates of autolysis were measured at low temperatures, around to 14 °C [38], On the other hand, as far as the influence of pH on the rate of autolysis was concerned, an optimum pH close to neutrality was observed on several occasions [39,40]. Optimum autolysis in the acidic range of pH was also reported [41].

Autolytic systems of several Gram-positive low GC% bacteria have been studied such as those found in *Staphylococcus aureus* [42,43], *Bacillus subtilis, Bacillus thuringiensis* [44], *Pediococcus* spp. [45], *Lactococcus lactis* [46], *Enterococcus faecalis* and *Lactobacillus pentosus* [47]. Several studies relate to autolysin genes in lactic acid bacteria particularly *Enterococcus faecalis* [48], *Enterococcus hirae* [49] and *Lactococcus lactis* [50] have been characterized [15,51,52], including the cloning and sequencing of the major lactococcal autolysin N-acetylmuramidase (AcmA) [50]. However, the use of genetically-modified starters in cheese is not always accepted and an alternative approach, consisting of the selection of natural starter strains with ideal autolytic properties, has been suggested [53].

Few studies have assessed the effect of physicochemical factors such as, salt concentration, pH and temperature on the autolysis of *Lactococcus lactis* by interacting them together at the same time, and examining the linear and quadratic effect of each of these factors and their possible interaction, which brings us to propose this alternative, whose objective is to intensification of this activity using a statistical optimization approach, which seems to be a judicious and interesting methodology that has not been or very little documented to our knowledge.

As an alternative to the one-factor-at-time, classical experimental approach, the experimental design method was used, in which the levels of all factors were varied at same time for each experiment. The advantages included a reduction in the number of trials, the ability to cover a large number of factors, the detection of interactions between factors, the detection of optima, a higher precision of the response data, and the empirical modeling of the data [54].

This optimization approach for any industrial interest response is based on the control, design and analysis of the developed mathematical models, formulated with parameters of clear biological significance and statistically consistent which can be easily implemented to miscellaneous applications. The objective of such statistically designed optimization study are to (i) confirm previous effects and interactions, (ii) estimate specific curvature or quadratic effects, and (iii) determine optimal settings of the critical factors. This strategy of optimization is a statistical Design of Experiment (DOE) method, which systematically evaluates more than one independent factor at a time. The Response Surface Methodology (RSM) is commonly used to explore nonlinear relationships between studied factors and the dependent variables [54,55].

Compared with conventional methods, the 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 [56,57]. Central composite design (CCD) [55] is an experimental strategy for seeking the optimum conditions for a multivariable system and it is an efficient technique for optimization. It also provides information about optimal values of these factors to determine the expected largest (or smallest) value for the dependent variable of interest. This methodology includes factorial design and regression analysis [54,55,58,59].

The Central Composite design (CCD) was the most used response surface design method, which had 5 levels and 3 levels, respectively for one numeric factor. In the present study, the RSM methodology has been applied and the coefficients of quadratic mathematical model were determined. The main purpose is to perform the Central Composite design (CCD) in order to seek the optimum levels of temperature, pH and NaCl concentration for a highest autolysis rate in a buffer system for *Lactococcus lactis* LCL strain.

Materials and methods

Bacterial strain and culture conditions. The bacterial strain *Lactococcus lactis* LCL, used throughout this investigation was obtained from the culture collection of "Laboratoire de Biologie des Microorganismes et Biotechnologie" of University of Oran 1 (Algeria). This strain was routinely maintained at 4 °C after growth at 30 °C for 12 or 24 h in M17 broth [60] implemented with glucose (G-M17) at the concentration of 1% (w/v). For longer-term maintenance, stock culture was stored at -20 °C in G-M17 broth with 20% (v/v) glycerol. As required, this culture was thawed and revitalized in G-M17 broth at 30 °C for 24h by two transfers.

Analytical methods. Autolysis rate (activity) of whole cells of *Lactococcus lactis* LCL strain in buffer solution was evaluated as described by Ostlie et al. [52]. The bacteria was harvested until late exponential growth phase (OD_{660nm} = 0.8 – 1.0) grown in G-M17 medium, washed twice in potassium phosphate buffer (50 mM, pH 6.5) and resuspended in the same buffer. The initial OD_{660nm} was adjusted at the start point (0.6–0.8) supplemented with appropriate concentration of NaCl according to the designed experiences. The samples were incubated at different temperatures according to the Central Composite Design (CCD) matrix (Table 1), and autolysis was monitored by measuring the decrease in OD_{660nm} after 72 h. The extent of autolysis was expressed as the percentage decrease of the optical density [61]. The essays were performed in triplicate.

Central Composite Design and Optimization using Response Surface Methodology. The statistically-based optimization using Central Composite Design (CCD) was carried out under response surface methodology (RSM), which is widely used in experiments where the main interest is in modeling the relationship between a number of quantitative factors and one or more response variables, then locating the combination of the factor levels that yields the best response [62]. 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) in their relation to the dependent variable.

A central composite design (CCD) for three independent variables – each at five levels (-α, -1, 0, +1, and +α) with eight cube points, six star points (± α = 1.68) and three replicates at the center points leading to 17 sets of experiments - was used to develop a second order polynomial model, which determined the optimal values of variables for the optimization of the autolytic activity of Lactococcus lactis LCL strain. Temperature, NaCl concentration and pH, were chosen as independent variables for investigation. The variables of the experiments were coded according to the following equation (Eq. 1).

$$x_i = (X_1 - X_0) / \Delta X \qquad i = 1, 2, \ldots\ldots, k \qquad \text{Eq. (1)}$$

where, x_i is the coded value of an independent variable, X_1 is the real value of an independent variable, X_0 is the value of X_1 at the center point, and ΔX the step change value.

Using the CCD method, a total of 17 experiments with various combinations of Temperature, pH and NaCl concentration were conducted. The minimum and maximum ranges of variables were used, and the full experimental design with respect to their coded values is shown in (Table 1). The second-order polynomial equation comprised linear, quadratic and interaction terms were used to calculate the predicted response, is shown below (Eq. 2):

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

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 and β_{ij} is the interaction coefficient and k is number of factors.

Statistical data analysis. STATISTICA v.7.0 software package (StatSoft, USA) was used to perform the multiple regression analysis and to generate the response surface plots of the obtained data to understand the interaction of different variables. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA) and the Fisher's test value (F-test) was employed to evaluate the statistical significance of the polynomial model equation (Eq. 2). The coefficient of multiple correlations R and the coefficient of determination R^2 were calculated to evaluate the performance of the regression equation. The significance of the regression coefficients was tested by a T-test. The optimum levels of the selected variables were obtained by solving the regression equation and also by analysis the response surface contour plots and desirability charts. All the experiments were conducted in triplicate and their mean values were used for analysis.

Experimental validation of the optimized conditions. In order to validate the optimization conditions for a desired level of response (autolysis), three tests were carried out at the optimal values of variables to confirm the results of the response surface analysis.

Results and Discussion

The effects of three above-mentioned variables on the autolytic activity were optimized using response surface optimization. Table 1 displays the design matrix of the obtained results with the respective values of variables in coded units and real from of the temperature of incubation (X_1), NaCl concentration (X_2) and pH (X_3), using the *Lactococcus lactis* LCL strain.

The highest rate of autolytic activity achieved in the verification experiment was 40 % (as seen in run 15), obtained around the center points, at 25°C, 35 g/l of NaCl and pH = 7 (Table 1). The results of the second-order response surface models for the autolytic activity in the form of analysis of variance (ANOVA) were given in Table (2) and (3) respectively. The regression equation (Y) demonstrated that the autolytic activity was an empirical function of test variables in coded unit as shown in following equation (Eq. 3).

$$Y = 38{,}9032 - 0{,}3109\, x_1 + 4{,}7568\, x_2 + 5{,}3948\, x_3 - 2{,}8060\, x_1^2 - 4{,}1994\, x_2^2 - 8{,}0748\, x_3^2 + 1{,}0653\, x_1x_2 - 0{,}1250\, x_1x_3 - 0{,}9602\, x_2x_3 \quad \text{Eq. (3)}$$

The quadratic model in Eq. (3) contains nine terms, three linear terms, three quadratic terms and three factorial interactions, in which Y is the predicted response (autolysis rate) and X_1, X_2 and X_3 are the coded values of the test variables; temperature of incubation, NaCl concentration and pH, respectively.

The model coefficients were estimated by multiple linear regressions and the significance of each coefficient was determined by Student's *t*-distribution and *P*-values, which is listed in Table 2. The P-value was used as a tool to check the significance of each of the coefficients. A larger magnitude of the *t*-test and smaller *P*-value denote greater significance of the corresponding coefficient.

The results show that the variables X_2 (NaCl) and X_3 (pH) had a significant linear effect on the response, based on *P*-values lower than 0.05. Moreover, as these variables have positive coefficients (Table 2), this means that an increase of NaCl concentration and pH in the system results an increase on the autolytic activity of *Lactococcus lactis* LCL strain. The same is observed with the squared variables X_1^2, X_2^2 and X_3^2, which are also significant with a negative effect. The negative signs of these terms bring about a scaling down of this response when their levels increase in the system. Whereas the linear effect of temperature (X_1) and the all interactions terms (X_1X_2, X_1X_3 and X_2X_3) were not significant in the range of this study, considering their *P*-value. The non-significant coefficients were discarded from the Eq. (3) leading to the following equation Eq. (4) representing the reduced fitted model.

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

Table 1. Box-Wilson central composite design (CCD) with three variables used for the optimization of autolytic activity of *Lactococcus lactis* LCL strain.

Runs	Coded level of variables			Actual level of variables			Autolytic activity (%)	
	X1	X2	X3	Temperature (°C)	NaCl (g/l)	pH	Experimental	Predicted
1	-1	-1	-1	15	10	4	14	13.96
2	+1	-1	-1	35	10	4	11	11.46
3	-1	+1	-1	15	56	4	19	23.27
4	+1	+1	-1	35	56	4	24	25.02
5	-1	-1	+1	15	10	10	27	26.92
6	+1	-1	+1	35	10	10	27	23.92
7	-1	+1	+1	15	56	10	32	32.38
8	+1	+1	+1	35	56	10	33	33.64
9	-1.68	0	0	8.2	35	7	34	31.73
10	+1.68	0	0	41.8	35	7	30	31.00
11	0	-1.68	0	25	0	7	21	23.43
12	0	+1.68	0	25	70	7	39	35.69
13	0	0	-1.68	25	35	2	11	8.00
14	0	0	+1.68	25	35	12	24	25.71
15	0	0	0	25	35	7	40	39.29
16	0	0	0	25	35	7	39	39.29
17	0	0	0	25	35	7	39	39.29

The statistical analysis of Eq. (4) was checked an *F*-test and the analysis of variance (ANOVA) for the quadratic response surface model were summarized in Table (3). This analysis demonstrates that the Eq. (4) was a highly significant model. It was evident from the Fisher's *F*-test (18,05) with a very low probability value (0.0004). The model's goodness of fit was also checked by coefficient of determination (R^2). The value of the coefficient of determination (R^2 = 0,9586) for Eq. (4) indicates that the sample variation for autolytic activity of 95,86% was attributed to the independent variables and only 4,14% of the total variation cannot 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 [63].

The value of the adjusted coefficient of determination (adj R^2 = 0,9055) was also satisfactory which indicates the good significance of model. The high *R* value (0.9516) also show a high degree of agreement of the experimental values with those predicted.

This correlation is also confirmed by the plot of predicted versus experimental values of the rate of autolytic activity in Figure. (1), as all points cluster around the diagonal line, demonstrating that no significant violations of the model were found.

The 3D response surface plot is generally the graphical representation of the regression equation. It is plotted to understand the interaction of the variables and locate the optimal level of each variable for maximal response (autolysis rate). Each response surface plotted represents the different combinations of two test variables at one time while maintaining the other variable at the zero level. The convex response surfaces suggest that there are well-defined optimal variables. If the surfaces are rather symmetrical and flat near the optimum, the optimized values may not vary widely from the single variable conditions [63].

Table 2. Model coefficients estimated by multiplies linear regression.

Factor	Coefficient	Standard Error of Coefficient	Computed t-Value	P-value
Intercept	38.9032	1.7428	22.3218	0.0000*
(1) Temperature (°C)(L)	-0.3109	0.8266	-0.3761	0.7179
Temperature (°C)(Q)	-2.8060	0.8968	-3.1288	0.0166*
(2)NaCl (g/l)(L)	4.7568	0.8585	5.5407	0.0008*
NaCl (g/l)(Q)	-4.1994	1.0449	-4.0187	0.0050*
(3) pH (L)	5.3948	0.8292	6.5053	0.0003*
pH (Q)	-8.0748	0.9088	-8.8847	0.0000*
1L by 2L	1.0653	1.0766	0.9894	0.3553
1L by 3L	-0.1250	1.0783	-0.1159	0.9109
2L by 3L	-0.9602	1.0766	-0.8918	0.4020

Table 3. Analysis of variance (ANOVA) for full quadratic model.

Source	Sum of Squares	Degrees of freedom	Mean of Square	*F*-test	*P*-value
Model	1454.84	9	161.65	18.05	0.004*
Residual Error	62.68	7	8.95		
Total	1517.52	16			

*Statistical significance: R2 = 0.9586 ; adj. R2 = 0.9055 ; R = 0.9791 ; adj. R = 0.9516

The graphics representation of response surface using Box-Wilson CCD shown in Figures (2) helps to visualize the effects of temperature of incubation, pH and NaCl concentration on the autolytic activity of *Lactococcus lactis* LCL strain.

The pH and NaCl concentration appear to be the variables dominant that control the autolysis rate of *Lactococcus lactis* LCL strain, accompanied with less involvement effect of temperature. The maximum autolysis rate was obtained at experiment number 15 at 40%, around central point region (Figure 2). The predicted response at these levels of variables was found at 39.29%, which is very close to the observed value.

The optimum levels of the tested variables were represented in desirability charts (Figure. 3), constructed using Response Surface Regression in STATISTICA software. These levels were as follows; the incubation temperature at 25 °C, NaCl concentration at 52.50 g/L and a neutral pH at 7 for a desirable prediction value of autolysis rate of 39.918%.

In order to confirm the adequacy and the validation of this model predicting a maximum rate of autolysis by *Lactococcus lactis* LCL strain, three additional experiments were also conducted at these predicted optimum levels. The mean value obtained is found to be 39%, which is in excellent agreement with the predicted value confirming the validity of the model.

In the present studies, significant variables were determined with only 17 experiments, using Box-Wilson central composite design (CCD). In this way, variables were reduced for optimization and a lot of time was saved. That said, in this kind of optimization study, all the factors were studied at the same time, which not only saves time, but also helped us to determine their interactions, that was not possible if we use one factor at a time technique. The prediction for maximum autolysis rate for the *Lactococcus lactis* LCL strain was also being possible due to this methodology.

Our observed value (40%) of autolysis rate was almost very close to that predicted (39.29%) at the calculated optimum levels. This small difference indicated that the range setting of variables was appropriate. The results obtained from this study may help us at further scale up studies of the autolytic activity.

Over the past decades, extensive work has been done to biotechnological processes able to increase many technology-related activities, including that of the autolysis of lactic acid bacteria. These bacteria contain a large array of intracellular enzymes, who's some of them involved in the production of flavor compounds during cheese ripening. However, *Lactococcus lactis* cell lysis must occur first, to allow interaction between bacterial enzymes and substrates present in the cheese matrix [18,19]. For this reason, bacterial lysis should be considered as an essential event during cheese ripening [64], and consequently, a production of these autolytic cultures in sight of an applications in cheese industry, desirable's autolysis conditions in downstream processes must be adjusted.

Despite it has been generally recognized that the environmental factors such as salt concentration, pH and temperature affects the bacterial cell lysis; there have been few studies undertaken to measure the effect of each of these factors and their possible interaction, on the autolysis of *Lactococcus lactis* using the RSM methodology.

Choosing the right type and levels of the environmental factors such as salt concentration, pH and temperature, appears to be very important impact on the autolysis of *Lactococcus lactis*. RSM has been proved to be a judicious and an inexpensive alternative, adopted for optimize the biotechnological processes. As far as we knew, this was the first trial to optimize the autolytic activity of *Lactococcus lactis* strain using the RSM methodology. In the present study, by using this method (RSM) involving the Central Composite Design (CCD), we have been successfully optimizing the factors affecting positively the autolysis rate of *Lactococcus lactis*.

Autolysis is the result of peptidoglycan hydrolases action on the bacterial cell wall, producing cellular lysis [65]. The major enzyme (autolysin) with lytic activity against peptidoglycan have been described in *Lactococcus lactis* is the N-acetiylmuramidase AcmA [24]. Autolysin may differ in their activity depending on environment conditions such as pH, salt concentration, water activity, ionic strength and temperature. Also, there are variations on the level of autolysis by different strains of *Lactococcus lactis* [24].

Boutrou et al., [64] reported wide variations of autolysis amongst 26 lactococci strains, incubated in buffered media (50mM sodium citrate + 0.25M NaCl, pH 5.0) for 14 days at 13 °C. These authors also classified the lactococci strains according to their lytic capacity into low (-15 to 0%), medium (0 to 15%) and high (15 to 30%). In the same way some many others authors, have determined the autolysis of many *Lactococcus lactis* strains in buffered media under different conditions. The treatment used by these authors is based on the usage of a combination of several buffer solutions with variable pH and NaCl concentrations [18,30,66,67].

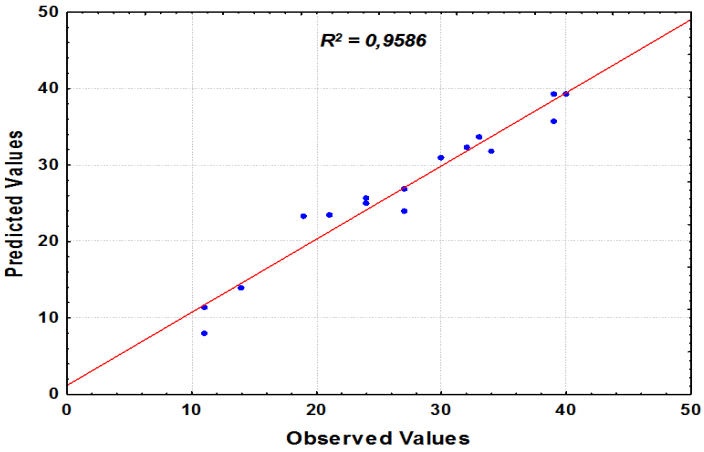

Figure 1. Plot of predicted vs. observed values of autolytic activity of *Lactococcus lactis* LCL strain

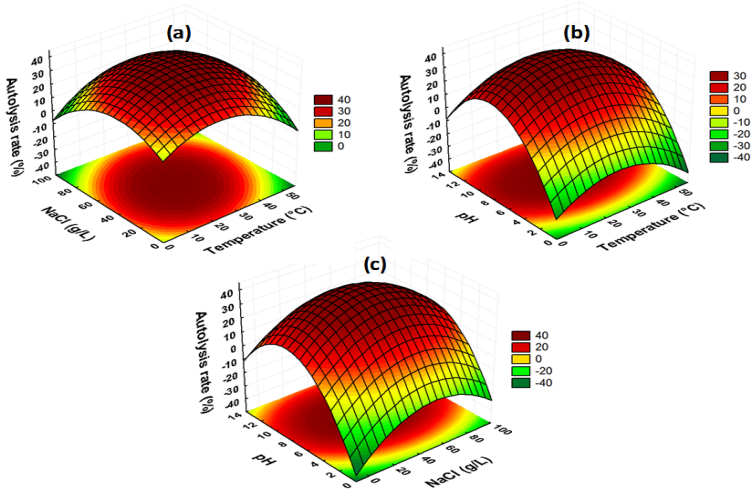

Figure 2. Response surface plot showing the effect of Temperature, pH and NaCl on autolytic activity of *Lactococcus lactis* LCL strain. (Each graphic represent the combinations of two test variables at one time while the other variable was fixed at the central point). a : [temperature] vs.[NaCl]; b: [pH] vs. [temperature] and c: [pH] vs. [NaCl].

Data collected shown large variation in the autolysis rate observed among the all strains of *Lactococcus lactis*, because some combinations seem to be to increase the autolysis of certain strains, while another are disadvantaged. Each strain of *Lactococcus lactis* had different behavior depending on the lysis solution used. In general, most strains of *Lactococcus lactis* had higher percentages of autolysis under acidic conditions and low salt concentration around pH 5 and 1% NaCl, but also at neutral pH and high salt content about pH 7 and 5% NaCl. In contrast, the acidic media with high salt content (pH 5 and 5% NaCl) did not favor the autolysis of most strains of *Lactococcus lactis* [18,30,66,67].

On the other hand, little is known about the effects of high temperatures on the metabolic activities of lactic acid bacteria, the case of enzymes responsible of glucose metabolism in lactobacilli, for cells grown at 51 and 53.5 °C, they seemed to be more active at high temperatures. In contrast, the lytic enzymes seemed to be less active at those higher temperatures than at 37 °C, as both PepX and autolytic activities were reduced at the higher temperatures [68].

NaCl is a well known triggering factor for autolysis [27,68,69] reported that autolysis was induced and influenced by the concentration and type of salt used (NaCl, KCl, K_2PO_4, Na2PO4), but not by high temperature or low pH. Husson-Kao et al.,[69] showed that pH values below 5 reduced autolysis of Streptococcus thermophilus whereas a temperature increase from 42 to 50 °C resulted in greatly increased autolysis.

Based on this kind of studies, the vast majority of authors suggests and concludes that triggering factors of autolysis in lactic acid bacteria are strongly specie/strain depended, not only for the percentage of autolysis presented by each strain, but also in response to the conditions used. This variability among *Lactococcus lactis* strains has been previously described in lactic acid bacteria among strains belonging even to the same species [22-24,27,64,68-70]. It has been suggested that variation of autolysis among strains is related with differences in cell wall composition and autolysins [24,28].

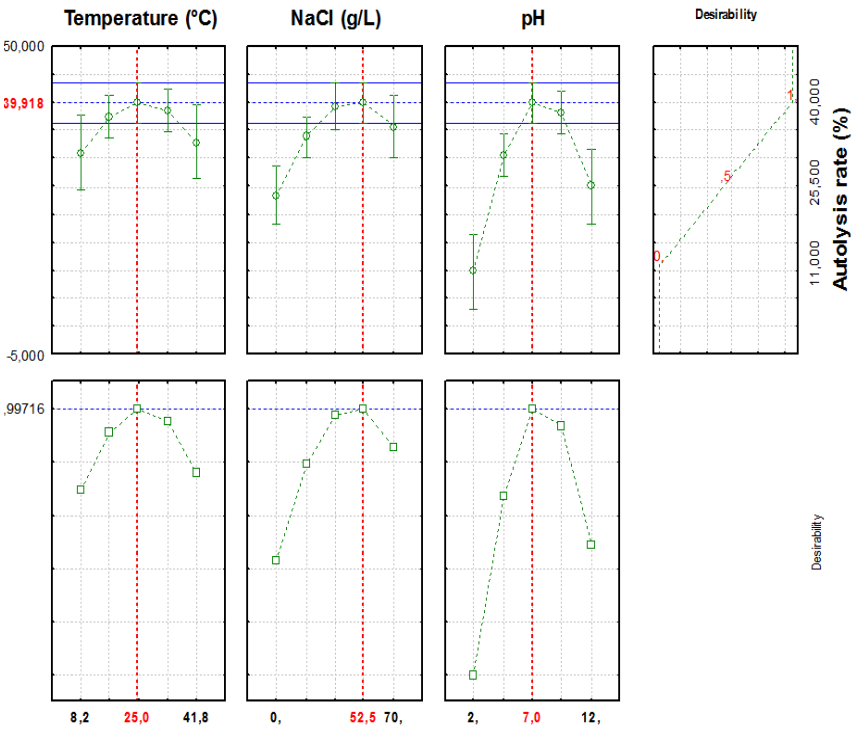

Figure 3. Profile of predicted values and desirability charts of independent variables for maximum response (autolysis rate)

Peptidoglycan is the bulwark of fortification of the bacterial cell; it is the major component of its cell-wall, giving it properties of extensibility, flexibility and elasticity and determining it cell shape and him providing resistance to internal osmotic pressure [71]. If the latter are hydrolyzed enzymatically by autolysins (mainly AcmA), they become soluble in water and lose their capacity for mechanical structure, thus producing cell lysis [72]. Changes in the electrochemical properties of the cell-wall have a direct effect on the adhesion of autolysins (mainly AcmA) and consequently on the hydrolysis of peptidoglicans [73]. Different pH and NaCl concentration conditions in the medium can cause changes in the electrochemical properties of the cell-wall of *Lactococcus lactis*, probably affecting the way autolysins interact with the cell-wall.

The effect of pH and salt on the rate autolysis of *Lactococcus lactis* remains confused, but likely related to a changes that operate in the cell wall as a response to environmental conditions like pH and ionic strength, as well as changes in the expression, activity and attachment to the cell wall of autolysins (AcmA). A change in pH and NaCl concentration can also generate contraction or expansion of the cell wall; increasing or decreasing cellular lysis. The enhancement of electrostatic interactions among charged peptidoglycan groups result in cell wall contraction; whereas repulsion forces result in cell wall swelling (expansion) [72].

The results obtained confirmed that the pH and salt content, as well as the temperature but at a lower level of contribution, have significantly affected the rate of autolysis of *Lactococcus lactis* LCL strain. According to our results, the maximum responses of autolysis can be achieved at neutral pH, and depending of the salt concentration in the media, included in the range of 34 to 52 g/L and around a temperature of 25 °C. This seems to be in perfect agreement with a large body of work carried out in this sense, but by tackling other methodological approaches. Characterization of *Lactococcus lactis* autolysis properties under different physicochemical variables, such as temperature, salt and pH conditions may facilitate a correct selection of appropriate strains according to the type of cheese to be produced. However, further information about cellular lysis *in situ* (in cheese or curd) of *Lactococcus lactis* under different salt and pH conditions is required and desirables.

Conclusion

The central composite design and response surface methodology enabled the determination of optimal operating conditions, this method it turned out to be a very efficient to sort the optimal levels of the variables for obtaining the maximum responses of autolysis of *Lactococcus lactis* LCL strain. The validity of the model was proven by fitting the values of the variables to the model equation and by carrying out experiments using these values. The optimization of the analyzed responses demonstrated that the best results of autolysis rate (39 to 40%) were obtained at 52 g/L of NaCl, pH 7 and at 25 °C. All points were located near the central point of the design. This also made it clear that the *Lactococcus lactis* LCL strain proves to be having great autolytic potential, mostly when put in the optimal conditions recommended.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

In this work the Mehiaoui's (MEHIAOUI Hanane) contribution editorial and experimental is the largest majority, as the latter represents a large part of his thesis work, succeeded by that of his supervisor of thesis, the Prof. ROUDJ Salima. Prof. HASSAINE Omar contributed in the supervision of the experimental and modeling parts of this work in addition to the writing and correction of the manuscript.

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