

Research Article

Canonical Analysis Technique as an Approach to Determine Optimal Conditions for Lactic Acid Production by *Lactobacillus helveticus* ATCC 15009

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The response surface methodology and canonical analysis were employed to find the most suitable conditions for *Lactobacillus helveticus* to produce lactic acid from cheese whey in batch fermentation. The analyzed variables were temperature, pH, and the concentrations of lactose and yeast extract. The experiments were carried out according to a central composite design with three center points. An empirical equation that correlated the concentration of lactic acid with the independent variables was proposed. The optimal conditions determined by the canonical analysis of the fitted model were 40°C, pH 6.8, 82 g/L of lactose, and 23.36 g/L of yeast extract. At this point, the lactic acid concentration reached 59.38 g/L. A subsequent fermentation, carried out under optimal conditions, confirmed the product concentration predicted by the adjusted model. This concentration of lactic acid is the highest ever reported for *Lactobacillus helveticus* ATCC 15009 in batch process using cheese whey as substrate.

1. Introduction

1.1. Cheese Whey as a Pollutant. Cheese whey is the liquid remaining after the precipitation and removal of milk casein during cheese making. This byproduct represents 85–90% of the milk volume and retains 55% of milk nutrients. Among the most abundant of these nutrients are lactose (4.5–5.0% w/v), soluble proteins (0.6–0.8% w/v), lipids, and mineral salts [1]. It is produced in large amounts by the dairy industry. To produce 1 kg of cheese are produced, on average, 9 kg of whey.

Because it is a byproduct of low economic value, formerly the cheese whey was simply dumped in watercourses without any previous treatment. This caused the whey to become a serious environmental problem due to its high content of organic matter, with BOD = 30,000–50,000 mg/L and COD = 60,000–80,000 mg/L [2].

1.2. Cheese Whey as a Resource. The whey produced in the dairy industry has two destinations: disposal or utilization. Disposal here means launching over the field, pumping in

water courses, and treatment in effluent systems. In the first case, the use of whey as a fertilizer can over time unduly increase soil salinity. The pumping of untreated whey in watercourses is an environmentally incorrect solution and it is banned in several countries.

Although it solves the legal problems associated with the launch in watercourses, treating whey as an effluent increases production costs and brings no economic return to the producer [3, 4].

The recovery of nutrients from whey and its transformation into higher value-added compounds are better alternatives than the disposal. Thus, it is possible to have a parallel economic activity to reduce the polluting effect. The organic component available in the whey can be used in various ways.

The proteins can be separated by ultrafiltration and used as food supplement and raw material to manufacture nutritional products [5]. However, the recovery of the proteins little contributes to the decrease of the polluting load of the whey, consisted mainly of the lactose present in the permeated. Therefore, the study of the several possibilities to

use this sugar is of great interest. One of the most promising alternatives is to use it as a source of low cost carbon, for the production of organic acids by fermentation. Most of the researches developed in the last years seek the production of lactic acid of high-added value, through the fermentation of the lactose present in the whey [6].

1.3. The Lactic Acid. Lactic acid is a versatile product that finds applications in several areas. It is used as acidulant in the food, cosmetics, and pharmaceutical industries [7]. Its isomers *L*(+) and *D*(-) can be polymerized to obtain compounds with different properties, depending on their intended application. In the medical area, the lactic acid has been used in the production of biodegradable polymers, used as scaffold in tissue transplants [8, 9]. The lactic acid polymers are also used in the production of biodegradable packages [10].

From the amount of lactic acid produced annually all over the world, about 90% is obtained by fermentation. The rest is synthesized starting from the hydrolysis of the lactonitrile. The production of lactic acid by fermentation presents advantages. One of them is the possibility to use renewable substrate, such as starch and cellulose. Another great advantage is that some strains can produce pure forms *L*(+) or *D*(-), while the chemical course always takes to the formation of a racemic mixture. The most commonly used substrates for the fermentative production of lactic acid are cheese whey, molasses, and starch. Before its use in fermentative processes, the whey must be deproteinized and demineralized. The separation of the proteins is usually made by ultrafiltration. Yeast extract, peptone, powder milk, and soybean meal are used as supplements in the fermentation of the whey. The microorganisms most frequently used are *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii*, and *Lactobacillus casei* [11].

1.4. Canonical Analysis. Nowadays, most of the studies over the fermentative production of lactic acid have the process optimisation as a goal. The experiments are designed to find better media for growth and fermentation, besides optimum values for process variables such as pH and temperature. Several researchers have been using the response surface methodology (RSM) to achieve these goals. Hujanen et al. [12] studied the optimisation of the production of lactic acid using *Lactobacillus casei*. The optimum operating conditions were obtained at 35°C and pH 6.3. Téllez-Luis et al. [13] and Bustos et al. [10] studied the optimisation of a low cost fermentation medium, made up of corn steep liquor supplemented with peptone and yeast extract. Naveena et al. [14] achieved the optimisation of a solid medium made with wheat bran to produce lactic acid using *Lactobacillus amylophilus*. However, the authors of all these works did not use the canonical analysis to find the point of maximum response.

Canonical correlation analysis is a multivariate statistical model that facilitates the study of interrelationships among sets of multiple dependent variables and multiple independent variables. Whereas multiple regression predicts a single dependent variable from a set of multiple independent

TABLE 1: Coded and actual values of the central composite design.

	X_i				
	- 1.55	-1	0	1	1.55
LC (g/L)	46.8	55	70	85	93.2
YE (g/L)	0	4.37	12.37	20.37	24.74
<i>T</i> (°C)	33.8	36	40	44	46.2
pH	4.5	5	6	7	7.5

variables; canonical correlation simultaneously predicts multiple dependent variables from multiple independent variables. Canonical correlation places the fewest restrictions on the types of data on which it operates. Because the other techniques impose more rigid restrictions, it is generally believed that the information obtained from them is of higher quality and may be presented in a more interpretable manner. For this reason, many researchers view canonical correlation as a last-ditch effort to be used when all other higher-level techniques have been exhausted. But in situations with multiple dependent and independent variables, canonical correlation is the most appropriate and powerful multivariate technique [15]. This technique has been used successfully in several studies on process optimization [16, 17]. It has gained acceptance in many fields and represents a useful tool for multivariate analysis, particularly as interest has spread to considering multiple dependent variables.

The objective of the present work was to use the response surface methodology and canonical analysis to find the most suitable conditions for *Lactobacillus helveticus* ATCC 15009 to produce lactic acid from cheese whey in batch fermentation. The influences of four process variables have been studied: temperature, pH, and the concentrations of lactose and yeast extract. *Lactobacillus helveticus* (ATCC 15009) has been chosen because it appears to be the most productive bacteria for lactic acid production from lactose [18]. The experiments were carried out according to a central composite design with three center points. The point of maximum response was obtained through a canonical analysis of the adjusted response surface [19].

2. Material and Methods

2.1. Inoculum and Fermentation Medium. *Lactobacillus helveticus* ATCC 15009 was supplied by the André Tosello Foundation (Campinas, SP, Brazil). Stock cultures were maintained on MRS broth [20] and deep frozen at -18°C. As required, these cultures were thawed and reactivated by two transfers in MRS broth (24 h, 37°C, 120 rpm). The cellular concentration of the inoculum was of 1.0×10^7 cells/mL.

The fermentation medium was made up of deproteinized reconstituted cheese whey, supplemented with yeast extract. The concentrations of lactose (LC) and yeast extract (YE) used are shown in Table 1.

2.2. Fermentations and Analyses. Batch fermentations were carried out at 150 rpm for 32 hours under anaerobic conditions in a 3.0 L fermentor NBS Bioflo 110 (New Brunswick Scientific, USA), in which 1800 mL of sterile culture medium

was inoculated with 200 mL of seed culture at 1.0×10^7 cells/mL. The pH was maintained at desired values by automatic addition of 6.0 N NaOH.

Lactic acid concentration was determined by a lactate analyzer, Accutrend Lactate (Roche, Germany). In this equipment, the lactic acid is measured by reflectance photometry, after a reaction between lactate and lactate-oxidase. The lactose was quantified using the 3,5-dinitrosalicylic acid method [21]. Biomass measurements were estimated from OD readings at 650 nm. The samples were centrifuged, washed twice with deionized water, and then rediluted, before being introduced in the spectrophotometer. By means of a calibration curve ($X = OD_{650}/2.35$), OD readings were converted to grams of dry cell per mL.

2.3. Experimental Design. The experiments were carried out according to a central composite design with three center points, leading to a set of 27 experiments. The independent variables were lactose concentration (LC), yeast extract concentration (YE), temperature (T), and pH. The chosen value for the extreme level of the design was $\alpha = 1.55$. This value was selected in order to obtain an orthogonal design, where the variance and covariance matrix is diagonal and the estimated parameters are not correlated amongst themselves. The coded (dimensionless) variables were defined as

$$x_1 = \frac{LC(\text{g/L}) - 70 \text{ g/L}}{15 \text{ g/L}}, \quad (1)$$

$$x_2 = \frac{YE(\text{g/L}) - 12.37 \text{ g/L}}{8 \text{ g/L}},$$

$$x_3 = \frac{T(^{\circ}\text{C}) - 40^{\circ}\text{C}}{4^{\circ}\text{C}}, \quad (2)$$

$$x_4 = \text{pH} - 6, \quad (3)$$

where x_1 , x_2 , x_3 , and x_4 are coded variables related to lactose concentration, yeast extract concentration, temperature, and pH, respectively. The levels used to code these variables are in Table 1. The variables range was selected on the basis of previous works [11].

2.4. Canonical Analysis. To quantify the effects of independent variables and their interactions, an empirical equation was fitted to the experimental data. This second order equation can be represented by matricial notation as

$$\hat{y} = b_0 + x'b + x'Bx, \quad (4)$$

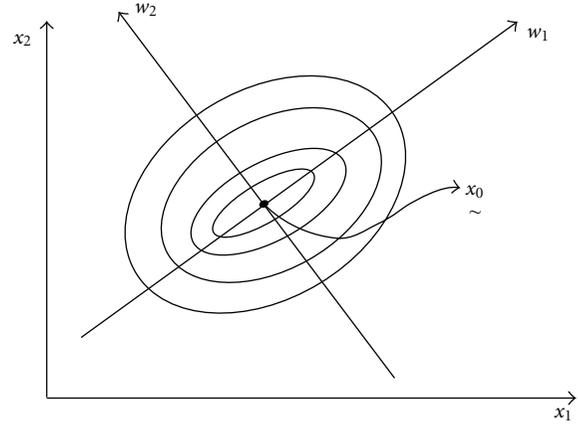


FIGURE 1: Graphical representation of axis translation.

where \hat{y} is the predicted response, $\mathbf{x} = \begin{bmatrix} x_1 \\ x_2 \\ \vdots \\ x_k \end{bmatrix}$ is the independent variables vector, $\mathbf{b} = \begin{bmatrix} b_1 \\ b_2 \\ \vdots \\ b_k \end{bmatrix}$ is the parameters vector of the first order terms, and

$$B = \begin{bmatrix} b_{11} & \frac{b_{12}}{2} & \dots & \frac{b_{1k}}{2} \\ \frac{b_{21}}{2} & b_{22} & \dots & \frac{b_{2k}}{2} \\ \dots & \dots & \dots & \dots \\ \dots & \dots & \dots & \frac{b_{k-1,k}}{2} \\ \dots & \dots & \dots & b_{kk} \end{bmatrix} \quad (5)$$

is the parameters matrix of the quadratic terms.

The expected maximum for the response (lactic acid concentration in this case), if it exists, will be a set of conditions (x_1, x_2, \dots, x_k) , such that the derivatives $\partial\hat{y}/\partial x_1, \partial\hat{y}/\partial x_2, \dots, \partial\hat{y}/\partial x_k$ are simultaneously zero. This value, say $\mathbf{x}_0 = [x_{1,0}, x_{2,0}, \dots, x_{k,0}]$, is the stationary point of the fitted surface. So, the derivative of \hat{y} with respect to the vector \mathbf{x} , equated to zero, is

$$\frac{\partial\hat{y}}{\partial\mathbf{x}} = \frac{\partial}{\partial\mathbf{x}} \left[b_0 + x'b + x'Bx \right] = \tilde{b} + 2B\tilde{x} = 0. \quad (6)$$

Thus the stationary point is

$$\mathbf{x}_0 = -B^{-1} \frac{\tilde{b}}{2}. \quad (7)$$

Here, the stationary point \mathbf{x}_0 (7) can be a point at which the fitted surface attains a maximum or a minimum, or a saddle point. The analysis of the nature of the stationary point begins with a translation of the response function from the origin ($x_1 = 0, x_2 = 0, \dots, x_k = 0$) to the \mathbf{x}_0 . Figure 1 shows a schematic representation of axis translation for two variables.

Due to the translation of the axes to stationary point \mathbf{x}_0 , (4) should be written in terms of the vector, \mathbf{z} , given by $\mathbf{z} = \mathbf{x} - \mathbf{x}_0$:

$$\begin{aligned}\hat{y} &= b_0 + (\mathbf{z}' + \mathbf{x}'_0)\mathbf{b} + (\mathbf{z}' + \mathbf{x}'_0)B(\mathbf{z} + \mathbf{x}_0) \\ &= b_0 + \mathbf{x}'_0\mathbf{b} + \mathbf{x}'_0B\mathbf{x}_0 + \mathbf{z}'\mathbf{b} + \mathbf{z}'B\mathbf{x}_0 + \mathbf{x}'_0B\mathbf{z} + \mathbf{z}'B\mathbf{z}.\end{aligned}\quad (8)$$

Since $\mathbf{z}'B\mathbf{x}_0 = \mathbf{x}'_0B\mathbf{z}$, and since the first three terms represent the response function evaluated at the stationary point, (8) is written as

$$\hat{y} = \hat{y}_0 + \mathbf{z}'(\mathbf{b} + 2B\mathbf{x}_0) + \mathbf{z}'B\mathbf{z} = y_0 + \mathbf{z}'B\mathbf{z}.\quad (9)$$

Equation (9) represents the response surface, translated to the new origin. Thus, there is an orthogonal transformation, $\mathbf{z} = M\mathbf{w}$, such that

$$\mathbf{z}'B\mathbf{z} = \mathbf{w}'M'BM\mathbf{w} = \lambda_1w_1^2 + \lambda_2w_2^2 + \dots + \lambda_kw_k^2,\quad (10)$$

where M is the orthogonal matrix $k \times k$ ($M'M = I_k$) and $\lambda_1, \lambda_2, \dots, \lambda_k$ are characteristic roots of the matrix B . The determination of matrix M is important because the transformation $\mathbf{w} = M'\mathbf{z}$ enables one to obtain the expression relating the original variables \mathbf{z}_i (as a result \mathbf{x}_i , since $\mathbf{z} = \mathbf{x} - \mathbf{x}_0$) to the canonical one, \mathbf{w}_i . M is the matrix of eigenvector associated with the characteristic roots λ_i .

Therefore, based on (9) and (10), the response function can be expressed in terms of new variables w_1, w_2, \dots, w_k , whose axes correspond to the principal axes of the contour system. The form of the function in terms of these variables is called the *canonical form* and is given by

$$\hat{y} = \hat{y}_0 + \lambda_1w_1^2 + \lambda_2w_2^2 + \dots + \lambda_kw_k^2.\quad (11)$$

The sign and magnitude of the characteristic roots λ_i can determine the nature of the stationary point. If $\lambda_i < 0$, a move in any direction from the stationary point results in a decrease in \hat{y} (see (11)). Therefore, the stationary point represents a point of maximum response for the fitted surface. On the other hand, $\lambda_i > 0$, \mathbf{x}_0 will be a minimum for the fitted surface. Finally, in the case where the λ 's differ in sign, the stationary point is a saddle point.

In this work, the canonical analysis was accomplished through a routine implemented using the software *Maple 7*. The regression parameters of the model (4) were estimated using the software *Statistica 7*.

3. Results and Discussion

3.1. Experimental Results and Fitted Equation. Table 2 shows the central composite experimental design and the results obtained for lactic acid production. The lactic acid concentration varied in a wide range, from 0.45 g/L in experiment 26 to 56.88 g/L in experiment 12. The results obtained at the central level show good reproducibility.

To obtain the equation that describes the response (lactic acid concentration) as a function of the independent variables, hypothesis tests were performed using the *t*-Student statistics to identify the significant parameters. The parameters, estimated by the least-square method, with significance level greater than 10% ($P > 0.1$) were neglected.

TABLE 2: First central composite design and experimental results.

Run	LC (g/L)	YE (g/L)	T (°C)	pH	Lactic acid (g/L)
1	55.0	4.37	36.0	5.0	1.67
2	85.0	4.37	36.0	5.0	7.20
3	55.0	20.37	36.0	5.0	1.28
4	85.0	20.37	36.0	5.0	13.50
5	55.0	4.37	44.0	5.0	1.12
6	85.0	4.37	44.0	5.0	12.87
7	55.0	20.37	44.0	5.0	5.67
8	85.0	20.37	44.0	5.0	8.46
9	55.0	4.37	36.0	7.0	9.90
10	85.0	4.37	36.0	7.0	16.38
11	55.0	20.37	36.0	7.0	37.08
12	85.0	20.37	36.0	7.0	56.88
13	55.0	4.37	44.0	7.0	7.20
14	85.0	4.37	44.0	7.0	19.58
15	55.0	20.37	44.0	7.0	37.80
16	85.0	20.37	44.0	7.0	43.65
17	70.0	12.37	40.0	6.0	37.22
18	70.0	12.37	40.0	6.0	36.94
19	70.0	12.37	40.0	6.0	37.15
20	46.8	12.37	40.0	6.0	12.87
21	93.2	12.37	40.0	6.0	42.13
22	70.0	0	40.0	6.0	23.94
23	70.0	24.74	40.0	6.0	27.81
24	70.0	12.37	33.8	6.0	26.19
25	70.0	12.37	46.2	6.0	9.18
26	70.0	12.37	40.0	4.5	0.45
27	70.0	12.37	40.0	7.5	48.15

The fitted equation, in matricial notation, is given by ($R^2 = 0.92$)

$$\hat{y} = b_0 + \mathbf{x}'\mathbf{b} + \mathbf{x}'B\mathbf{x},\quad (12)$$

where

$$\mathbf{x} = \begin{bmatrix} x_1 \\ x_2 \\ x_3 \\ x_4 \end{bmatrix}, \quad \mathbf{b} = \begin{bmatrix} 5.57 \\ 6.56 \\ 0 \\ 12.35 \end{bmatrix},\quad (13)$$

$$B = \begin{bmatrix} -3.41 & 0 & 0 & 0 \\ 0 & -2.99 & 0 & 3.44 \\ 0 & 0 & -7.51 & 0 \\ 0 & 3.44 & 0 & -4.75 \end{bmatrix}.$$

Vector \mathbf{b} shows that the linear effect of temperature was not significant ($b_3 = 0$). The only significant interaction occurred between yeast extract concentration and pH ($b_{42}/2 = b_{24}/2 = 3.44$, matrix B). All quadratic

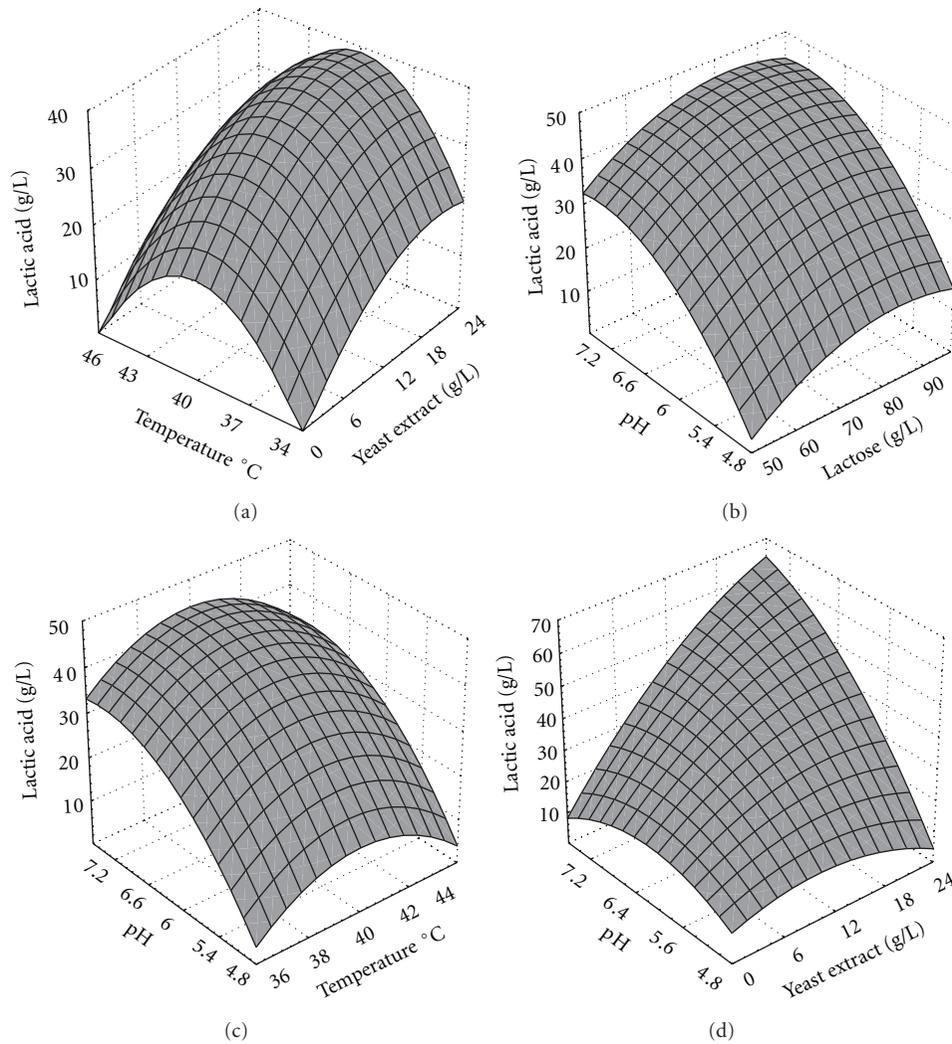


FIGURE 2: Response surfaces for the production of lactic acid as a function of the variables: (a) temperature and yeast extract concentration; (b) lactose concentration and pH; (c) temperature and pH; (d) yeast extract concentration and pH. For each surface, the other variables are in the center point.

effects, including the temperature, were significant ($b_{ii} \neq 0$, matrix B).

The influences of the independent variables and their interactions on the lactic acid concentration can be also analyzed through the response surfaces (Figure 2) obtained from (11). The production of lactic acid was maximum when the temperature was around 40°C ($x_3 = 0$), as it can be seen in Figures 2(a) and 2(c). The effect of temperature on the production of lactic acid by *Lactobacilli* has only been studied in a few reports [11]. *Lactobacilli* are mesophilic, which makes plausible the result obtained in this work, although some strains of *Lactobacillus helveticus* grow at 50–52°C [22]. The fact that the optimum temperature is close to 40°C can be considered acceptable when compared with the works available in the literature using cheese whey as substrate and strains of *Lactobacillus helveticus*. Tango and Ghaly [18], while studying the effect of temperature on product formation, performed experiments at 23°C, 37°C, and

42°C. The highest concentration of lactic acid was obtained at 42°C. Kulozik and Wilde [23] performed experiments at five levels of temperature: 35°C, 38°C, 42°C, 45°C, and 49°C. The product formation was slightly higher at 45°C than at 42°C. Norton et al. [24], Amrane and Prigent [25], and Schepers et al. [26] used a temperature of 42°C in their work, which corroborates the results obtained here, although they do not justify their choices.

Figure 2(b) shows that the lactic acid concentration was maximum when the lactose concentration was in the range of 70–85 g/L ($0 < x_1 < 1$). The response decreased for larger concentrations, probably due to the inhibition by the substrate. Several studies showed that cellular growth and lactic acid production by bacteria of the *Lactobacillus* genus are inhibited by the substrate [27, 28]. Tango and Ghaly [29] report that the growth of *Lactobacillus helveticus* and the production of lactic acid were inhibited for lactose concentrations greater than 80 g/L, which confirms

the results of this work. However, Aeschlimann and von Stockar [30] published a similar study where there was no inhibition by the substrate for initial lactose concentrations smaller than 132 g/L.

The strong influence of the pH on the process can be seen in Figures 2(b) and 2(c). The amount of lactic acid produced when the pH was smaller than 6 ($x_4 < 0$) was very little. The major influence of pH over lactic acid production in fermentation processes is due to the fact that the catalytic activity of the enzymes and metabolic activity of the microorganism depend on extracellular pH. According to Hofvendahl and Hahn-Hägerdal [11] and Wood and Holzapfel [22], optimal pH for lactic acid production by lactic acid bacteria varies between 5.0 and 7.0 and is dependent on the strain.

For values of pH equal or larger than 6 ($x_4 > 0$), there was higher production of lactic acid, except when the yeast extract concentration was smaller than 12.37 g/L ($x_2 < 0$). That interaction between pH and yeast extract is shown in Figure 2(d). Even in favorable conditions of pH, low concentrations of yeast extract led to low product concentrations. This happened due to the complex nutritional demands of the genus *Lactobacillus* [22]. The source of nitrogen is the main factor influencing the growth of these microorganisms. Because the fermentative production of lactic acid is associated to the cellular growth, there is no product yield if the medium does not have an appropriate concentration of nitrogen to promote growth [31]. According to Figure 2(a), the optimum concentration of yeast extract seems to be in the range from 20.37 g/L to 24.74 g/L ($1 < x_2 < 1.55$). This result is in accordance with Amrane and Prigent [25]. They carried out a study on the influence of the yeast extract concentration on the homolactic fermentation of cheese whey, using *Lactobacillus helveticus*. The experiments used yeast extract concentrations in the range 2–30 g/L. According to the authors, larger concentrations of this substance led to the same final concentration of lactic acid; however, the fermentation time decreased significantly. In this work, the production of lactic acid was very low at concentrations of yeast extract smaller than 12.37 g/L, for the fermentation time chosen for that study. Several authors have been looking for low cost nitrogen sources substitutes for yeast extract. Corn steep liquor seems to be a viable alternative [32–34]. Fungi and yeasts [8, 35] and adapted strains of *Lactobacillus* [29] have also been used with the same purpose.

3.2. Canonical Analysis. The point of maximum lactic acid concentration was determined through a canonical analysis of the fitted model (12). The stationary point (\mathbf{x}_0) obtained from (6) is given by

$$\mathbf{x}_0 = \begin{bmatrix} 0.82 \\ 15.54 \\ 0 \\ 12.56 \end{bmatrix}. \quad (14)$$

TABLE 3: Coded and actual values of variables in the second central composite design.

	X_i				
	-1.40	-1	0	1	1.40
YE (yeast extract concentration, g/L)	18.36	20	24	28	29.64
pH	5.6	6	7	8	8.4

The characteristic roots (λ_i) of matrix B (11) are all negative: $\lambda_1 = -7.51$, $\lambda_2 = -7.42$, $\lambda_3 = -3.41$, $\lambda_4 = -0.32$. Therefore, \mathbf{x}_0 is a point of maximum response.

From stationary point (12), it can be seen that the optimum coded value obtained for lactose concentration was $x_1 = 0.82$; this value corresponds to 82.3 g/L (see (1)). There are no reports about the ideal concentration of this substrate in the fermentative production of lactic acid using *Lactobacillus helveticus*. In works published on this subject, initial concentrations were in the range 50–150 g/L. Increases of the initial concentration of lactose to values greater than 100 g/L implied in greater product concentrations [26]. However, they caused a decrease of conversion from substrate to product $Y_{p/s}$ and, consequently, an increase in the concentration of residual lactose. Therefore, the value found in the present work can be considered plausible. The optimum coded value obtained for temperature was $x_3 = 0$, corresponding to 40°C (2), confirming the value indicated by the response surface analysis (Figures 2(a) and 2(c)).

The optimum values of yeast extract concentration ($x_2 = 15.54$) and of pH ($x_4 = 12.56$) extrapolated the limits of the experimental design (-1.55 to +1.55). Hence, a new experimental design had to be performed to find the best conditions for these two variables. In this new experimental design, the lactose concentration and temperature were set at the best conditions, that is, 82 g/L and 40°C, respectively. The coded variables for this second central composite design were defined as

$$x_2 = \frac{\text{YE(g/L)} - 24 \text{ g/L}}{4 \text{ g/L}}, \quad (15)$$

$$x_4 = \text{pH} - 7.$$

The coded and actual levels of these variables are in Table 3. The results of the new experimental design are showed in Table 4.

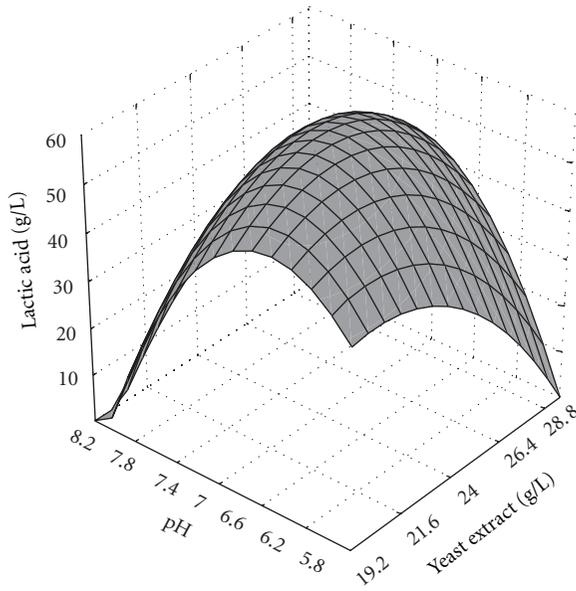
As before, an empirical equation was fitted to this new set of experimental data. The fitted equation ($R^2 = 0.95$) in matricial notation is given by

$$\mathbf{x} = \begin{bmatrix} x_2 \\ x_4 \end{bmatrix}, \quad \mathbf{b} = \begin{bmatrix} 0 \\ -6.77 \end{bmatrix}, \quad B = \begin{bmatrix} -0.16 & -0.05 \\ -0.05 & -0.06 \end{bmatrix}. \quad (16)$$

The response surface corresponding to this new equation is shown in Figure 3. The production of lactic acid was very small both for pH greater than 8 ($x_4 > 1$) and for yeast extract concentrations larger than 28 g/L ($x_2 > 1$). On the other hand, the product concentration was maximum for yeast

TABLE 4: New central composite design and experimental results.

YE (g/L)	pH	Lactic acid (g/L)
20	6	42.15
28	6	26.32
20	8	6.80
28	8	39.82
24	7	58.23
24	7	59.14
18.36	7	48.73
29.64	7	38.04
24	5.6	28.02
24	8.4	5.20

FIGURE 3: Response surface for lactic acid production as a function of the pH and of the yeast extract concentration ($T = 40^{\circ}\text{C}$, lactose 82 g/L).

extract concentrations in the range 20–24 g/L ($0 < X_2 < 1$) and pH around 7 ($x_4 = 0$).

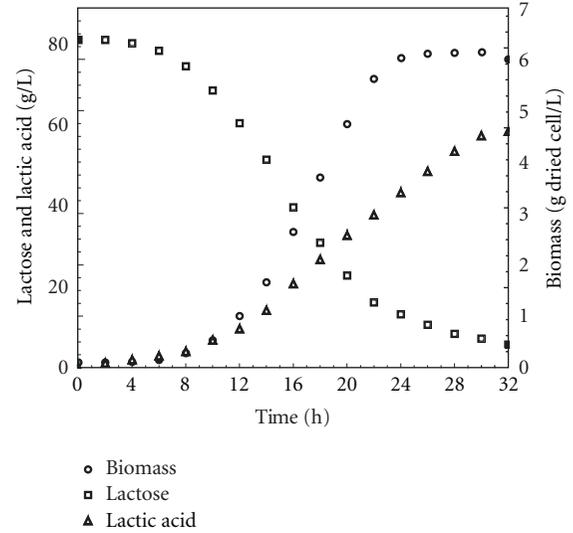
The point of maximum response was determined through a canonical analysis of the fitted model. The stationary point is given by

$$\mathbf{x}_0 = \begin{bmatrix} -0.16 \\ -0.20 \end{bmatrix}. \quad (17)$$

The actual values of the coded independent variables were obtained from (15). The coded variables $x_2 = -0.16$ and $x_4 = -0.20$ correspond to 23.36 g/L of yeast extract concentration and pH 6.8, respectively. With the new stationary point, the canonical form of the fitted model is given by

$$Y = 59.38 - 23.79w_2^2 - 5.61w_4^2. \quad (18)$$

This equation shows that both characteristic roots (λ_i) have negative sign. This means that any displacement from

FIGURE 4: Concentrations of product, substrate, and biomass as a function of the time, under the best conditions (lactose 82 g/L; yeast extract 23.36 g/L; temperature 40°C ; pH 6.8).

the stationary point causes a decrease of the response and, therefore, the stationary point is a maximum point. As a result, the optimum conditions determined by this study are temperature 40°C , pH 6.8, concentration of lactose 82 g/L, and concentration of yeast extract 23.36 g/L. Under these conditions, the canonical analysis predicts a production of acid lactic of 59.38 g/L.

A new fermentation was accomplished using the optimum operational conditions listed above. The result is shown in Figure 4. The concentration of lactic acid reached 59.15 g/L, confirming the result predicted by the canonical analysis. This value is the biggest ever reported in the literature for works that used cheese whey as substrate and strains of *Lactobacillus helveticus* ATCC 15009. The low residual concentration of the lactose, 5.75 g/L, shows that the substrate was almost totally consumed after 32 hours of fermentation. The cellular concentration reached the maximum value of 6.14 g/L. The factors of conversion from substrate into product $Y_{P/S}$ and from substrate into biomass $Y_{X/S}$ were 0.77 and 0.08, respectively.

4. Conclusions

This study demonstrated that the response surface methodology associated with the canonical analysis was appropriate to find the conditions that maximized the production of lactic acid from cheese whey fermentation. The optimal conditions determined by the canonical analysis were 40°C , pH 6.8, 82 g/L of lactose, and 23.36 g/L of yeast extract. At this point, the lactic acid concentration reached 59.15 g/L, the biggest value ever reported in studies using cheese whey as substrate and strains of *Lactobacillus helveticus* ATCC 15009 in batch fermentation.

The production of lactic acid was strongly linked to the pH and to the yeast extract concentration. The decrease of

the response for higher lactose concentrations indicates an inhibition by the substrate.

The high concentration of yeast extract at the point of maximum response justifies the search for low cost nitrogen sources.

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