Brazilian Journal
of Chemical
Engineering

ISSN 0104-6632
Printed in Brazil
www.abeq.org.br/bjche

Vol. 30, No. 02, pp. 267 - 276, April - June, 2013

IMMOBILIZED *Lactobacillus acidophilus* PRODUCED FROM WHEY AND ALGINATE

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(Submitted: May 16. 2011 ; Revised: April 23, 2012 ; Accepted: July 23, 2012)

Abstract - An analysis was made of the use of whey fermentation by *Lactobacillus acidophilus LA-5* for encapsulated probiotic bacteria cell production. Fermentation was done in a 2-liter Biostat B Fermentor at 28±1 ºC without air supply and agitation maintained at 200 rpm. Different processing conditions were studied using Center Composite Design applied to Surface Response Methodology. Maximum cell yield (2.7×10^{10} NMP/mL for 36 hours) was achieved with 30.85 g/L of lactose, a pH value of 6.45 and 1.04 g/L of inoculum. Cell growth was evaluated using reconstituted and fresh whey after 144 hours of fermentation in pre-optimized conditions. Cell concentration after fermentation was 10^{10} MPN/mL in all the assays. The Verhulst model proved to be satisfactory to fit the experimental results, providing a stationary cell concentration of 6.0 g/L and a specific growth rate of 0.09 h^{-1}. Cells were collected by centrifugation at 15000g for 5 minutes at 4 °C, immobilized in 2% alginate, and dried to a constant weight at 50 °C. Immobilized probiotic cells presented 10^{11} MPN/g, a time required to kill 90% of the organisms (D value) of 18 h (70 ºC), an activation energy of 76.04 kJ/mol for thermal inactivation, and an *in vitro* resistance to low pH (D value of 62.5 min at 37 ºC, pH 2.5).
Keywords: *Lactobacillus acidophilus*; Immobilized cells; Probiotics; Whey.

INTRODUCTION

Probiotics are live microorganisms that are beneficial to the host's health when administered in adequate amounts. In animal feed, probiotics reduce the need for therapeutic chemicals and antibiotics and increase overall health as well as food safety. In humans, probiotics are involved in defense against infection, prevention of cancer and in stabilizing or reconstituting the physiological balance of the intestinal microbiota (Wohlgemuth *et al.,* 2010; Oelschlaeger 2010; Aragon-Alegro *et al*., 2007; Balcázar *et al*., 2006; Fuller 1989). According to reports in the literature (Chávarri *et al*., 2010; Cruz *et al*., 2007; Talwalkar and Kailasapathy 2004), the minimum level of microbial cells to claim probiotic status in humans is 10^6 to 10^7 cells/g of probiotic product. Similar values of probiotics are suggested for cats, dogs and ruminants (Marshall-Jones *et al*., 2006; Baillon *et al*., 2004; Chaucheyras-Durand *et al*., 2010).

The genus *Lactobacillus* contains several species of probiotic bacteria that are natural inhabitants of the human gut and are found in a wide range of environmental niches. They can colonize the intestinal tract of humans and other mammals as a probiotic. In this genus, *Lactobacillus acidophilus* is the traditional species used in probiotics (Magalhães

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et al., 2008; Apás *et al*., 2008). Cruz *et al*. (2007) and Talwalkar and Kailasapathy (2004) highlight the fact that *Lactobacillus acidophilus* requires an environment of low oxygen tension for optimal growth and has complex nutritional needs related to the assimilation of free amino acids, peptides, vitamins, nucleotides and certain minerals.

One of the critical factors in the maintenance of probiotic cell viability is the choice of fermentation medium. Whey is an important fermentation medium constituent because of its nutritional value and year-round availability. The processing of milk to produce cheese generates cheese whey, which represents a source of water pollution if discarded. The world-wide production of this byproduct is estimated to be over 176 million tons per year and its pollutant load corresponds to a polluting power ten-fold greater than domestic sewage, with a Chemical Oxygen Demand (COD) between 40000 and 60000 $mg.L^{-1}$ (Ozmihci and Kargi, 2008; Tamime, 2006; Prazeres *et al*., 2012; Rech and Ayub, 2006).

Another critical factor is the maintenance of cell viability during probiotic production, storage and digestion by enteric or pancreatic enzymes. This factor can be regarded as a major limiting factor in commercial probiotic production. Drying and immobilization are both effective methods used to preserve probiotic activity during the storage period, and cell immobilization has also been shown to improve resistance to digestion by enteric or pancreatic enzymes. Other advantages related to the use of immobilized cells include increased cell density and a physical barrier against the high temperatures, high osmotic pressure and high levels of oxygen of the external environment generated during the production of probiotic formulations (Pan *et al*., 2009; Rayment *et al*., 2009; Meng *et al*., 2008; Cruz *et al*., 2007; Capela *et al*., 2007; Kailasapathy, 2006; Selmer-Olsen *et al*., 1996).

Immobilization using alginate has the advantage of being economical because of its low cost and simplicity: adding an alginate solution to a solution containing a mixture of calcium chloride can result in the formation of alginate beads with pore sizes of less than 17 nm. Alginates, which are natural anionic polysaccharides composed of D-mannuronic and L-guluronic acid residues joined linearly by (1–4)-glycosidic linkages, are also an accepted food additive and can be used safely in foods (Chávarri *et al*., 2010; Annan *et al*., 2008; Chan and Zhang 2005; Krasaekoopt *et al*., 2004).

The primary objective of this study was to analyze the use of whey fermentation to produce probiotic formulations containing *Lactobacillus acidophilus* cells. The secondary objective was to evaluate the stability of solid formulations containing these cells immobilized in dried alginate-calcium beads.

MATERIALS AND METHODS

Microorganisms

A lactic culture of *Lactobacillus acidophilus* La-5 was maintained at 5 ºC and replicated every 15 days (30 ºC, 24 h) in a selective MRS (Man, Rogosa and Sharpe) medium composed of 10 g/L peptone casein, 10 g/L meat extract, 5 g/L yeast extract, 20 g/L glucose, 1 g/L Tween 80, 2 g/L K_2HPO_4, 5 g/L CH_3COONa, 2 g/L ammonium citrate, 0.2 g/L $MgSO_4.7H_2O$ and 0.05 g/L $MnSO_4.H_2O$.

The cell concentration was measured in assays to determine dry cell concentrations and viable cell numbers. The dry cell concentration was measured as follows: the cells were harvested by centrifugation of 25 mL of cultivated medium (15,000g for 15 min) and dried at 90 ± 1 ºC until no change in weight was detectable. The viable cells were obtained by the Most Probable Number (MPN) methodology using MRS medium, as described by Man (1960). This methodology (MPN) was chosen because the quantification of cells in isothermal conditions at 35, 40, 60 and 70 ºC, described below, produces a large reduction in cell number and MPN has higher sensitivity for the extremely low level microbial counting (Sun *et al*., 2006; DiGeronimo *et al*., 1978).

Lactose and Protein Concentration

Lactose concentration was determined by a spectrophotometric assay with glucose oxidase (Leary *et al*., 1992) and the mass balance described by $L=1.9(G_2-G_1)$, where L represents the lactose concentration (g/L) and G_1 and G_2 are the glucose concentrations (g/L) before and after acid hydrolysis (20 µL 11.5 N HCl, 90 ºC for 5 min). Reducing sugar was also measured by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). The protein concentration was determined by the Lowry protein assay (Lowry, 1951).

Experimental Design

Optimization of *Lactobacillus acidophilus* production was attempted using Fractional Factorial

Design (FFD) with three replications at the central point and six axial points in a 1.8 liter BioStat B Fermentor at 28 ± 1 ºC without air supply and with agitation maintained at 200 rpm. Three factors were selected for use in designing the experiment: lactose concentration (X_1), pH (X_2) and inoculum size (X_3). The ranges of the variables investigated in this study were: 20 – 40 g/L for lactose, 5 – 8 for pH and 0.5 – 1.5 g/L for inoculum size. To create a response surface, the experimental data obtained from the above design were fitted to the following second order polynomial equation:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_1^2 + A_5X_2^2 + A_6X_3^2 + A_7X_1X_2 + A_8X_1X_3 + A_9X_2X_3 \quad (1)$$

where Y represents the expected *Lactobacillus acidophilus* cell concentration (MPN/mL), X_1 the lactose concentration, X_2 the pH and X_3 the inoculum size, and A_i with i=1,2,...,9 the adjustable parameters for fitting the data.

Characterization of *Lactobacillus acidophilus* Kinetics

Growth curves were determined for both reconstituted whey and fresh whey using the same reactor employed for the optimization studies with the same conditions of optimization for 144 hours of fermentation. Sampling was done at regular intervals to determine the viable cell counts and the dry cell, protein, and lactose concentrations.

To quantify the growth of microorganisms and to compare fermentation characteristics, the growth curves were adjusted to kinetic models (see Table 1) using two program codes implemented in Scilab 5.0.1 software and the statistical differences between the parameters of reconstituted whey and fresh whey were found by Student's t-test with a 95% confidence interval (p=0.05). One of the programs used to determine the parameters was the generic optimization function that evaluates gradients by finite differences (NDcost, scilab-5.2.2, Copyright © 1989-2010. INRIA ENPC); the other program used the Monte Carlo method (Taha, 2006).

Immobilization and Characterization of Immobilized Cells

Cell immobilization was performed in calcium alginate gel in the form of beads. The beads were prepared by placing a blend of sodium alginate (1%) and 10^8 cell/mL of harvested cells (15,000 g for 15 min) in a calcium chloride solution (0.2 mol/L). The beads were then dried at 50°C and evaluated by image processing using Image Pro Plus 7.0 software (Media Cybernetics).

Table 1: Growth Models

Model	Equation	
Monod	$\frac{dX}{dt} = \frac{\mu_M S}{S + K_S} X$ $\frac{dS}{dt} = \frac{-1}{Y_{\frac{X}{S}}} \frac{dX}{dt} - mX$	(2)
Verhulst	$\frac{dX}{dt} = \mu_M X \left(1 - \frac{X}{X_M}\right)$ $\frac{dS}{dt} = \frac{-1}{Y_{\frac{X}{S}}} \frac{dX}{dt} - mX$	(3)
Contois	$\frac{dX}{dt} = \frac{\mu_M S}{S + BX} X$ $\frac{dS}{dt} = \frac{-1}{Y_{\frac{X}{S}}} \frac{dX}{dt} - mX$	(4)

Note: X indicates the cell concentration (g/L), S is the lactose concentration (g/L), $Y_{X/S}$ is the stoichiometric coefficient (g cell/g lactose), μ_M is the optimum specific growth rate (h^{-1}), t is time (h), B is the Contois saturation constant (g lactose/g cell), and m is the specific maintenance rate (h^{-1}).

Cell viability and stability were also evaluated. The stability of probiotic cells in gastric conditions was simulated to determine the number of surviving cells in acid conditions (37 ± 1 ºC, pH 2.5 ± 0.1) after 30, 60 and 90 min of incubation, using the model described by Equation (5),

$$\log_{10} S(t) = -t / D \quad (5)$$

where t is time, S(t) is the survival rate, which is defined as the ratio between the number of survivors after exposure time t, N(t), and the initial number, No, and D is the time required for one log reduction in the number of cells (D value).

The stability was also investigated in accelerated storage tests using isothermal conditions at 35, 40, 60 and 70 ºC (Mitic *et al*., 1974). The survival curves were adjusted to the survival models presented in Table 2.

Table 2: Survival Models (Bailey, Ollis, 1986)

Model	Differential form		Integrated form	
First order	$-dN/dt = kN$	(6)	$N(t) = N_0 \exp(-kt)$	(7)
Logistic	$-dN/dt = k_1 tN + k_2 N(N_0 - N)$	(8)	$N(t) = \frac{N_0(k_1 + k_2 N_0)}{N_0 k_2 + k_1 \exp((k_1 + N_0 k_2)t)}$	(9)
Weibull	$-dN/dt = knNt^{n-1}$	(10)	$N = N_0 \exp(-kt^n)$	(11)
t^2	$-dN/dt = ktN$	(12)	$N = \frac{N_0 \exp(-kt^2)}{2}$	(13)

Note: N is the cell concentration (MPN/mL), t is time, k is the reaction rate constant in the Arrhenius equation k=ko.exp(-Ea/R.T), T is temperature, and Ea is the activation energy, R=8.314 J/(mol.K)

RESULTS AND DISCUSSION

Experimental Design

Table 3 shows the trials and results of the factorial design at 36 hours of fermentation. Cell concentrations ranged from 8.1×10^7 MPN/mL (trial 3) to 3.5×10^{10} NMP/mL (trial 16). As can be seen, the best results were found at the central point (Trials 15, 16 and 17): $(3.2 \pm 1.3) \times 10^{10}$, $(3.5 \pm 1.7) \times 10^{10}$ and $(3.0 \pm 1.1) \times 10^{10}$ NMP/mL.

The mathematical model established by Equation (1) with statistically significant parameters at the 90% confidence level (Student's *t* test) are presented in Equation (14). The coefficient of determination (R^2) was 87% meaning the model explained 87% of the variation in the response variable. The optimum cell concentration was found at a pH of 6.45 and concentrations of 30.85 g/L (lactose) and 1.04 g/L (inoculum size). These results show qualitative agreement between the theoretical and experimental cell concentration.

$$Y = 2.894 \times 10^{10} - 8.514 \times 10^9 X_1^2 - 1.124 \times 10^{10} X_2^2 - 9.956 \times 10^9 X_3^2 \quad (14)$$

Figure 1 presents the effect of lactose concentration and inoculum size on the viable cell concentration with the pH kept at the central point (pH 6.5), while Figure 2 illustrates the effect of inoculum size and pH on the viable cell concentration with the lactose concentration held at the central point (30 g/L). Note that the viable cell concentrations are optimized for a lactose concentration of 27 to 35 g/L, inoculum size of 0.8 to 1.2 g/L, and pH of 6.0 to 7.0.

The experimental results revealed an optimized viable cell concentration of about 10^{10} MPN/mL and cell concentrations of 6.0 ± 0.7 g/L. The cell concentration was about 1.38-fold higher than that reported in the literature (Tari *et al*., 2009).

Table 3: Study of lactose concentration, pH and inoculum size (factorial design)

Trial	X_1 (g/L)	X_2	X_3 (g/L)	MPN/mL
1	-1 (20)	-1 (5.0)	-1 (0.5)	$(9.2 \pm 2.0) \times 10^7$
2	-1 (20)	-1 (5.0)	+1 (1.5)	$(5.2 \pm 2.3) \times 10^8$
3	-1 (20)	+1 (8.0)	-1 (0.5)	$(8.1 \pm 3.1) \times 10^7$
4	-1 (20)	+1 (8.0)	+1 (1.5)	$(1.5 \pm 2.6) \times 10^8$
5	+1 (40)	-1 (5.0)	-1 (0.5)	$(6.0 \pm 1.7) \times 10^8$
6	+1 (40)	-1 (5.0)	+1 (1.5)	$(3.5 \pm 3.2) \times 10^9$
7	+1 (40)	+1 (8.0)	-1 (0.5)	$(7.9 \pm 1.5) \times 10^8$
8	+1 (40)	+1 (8.0)	+1 (1.5)	$(4.0 \pm 3.3) \times 10^9$
9	+α (16.4)	0 (6.5)	0 (1.0)	$(6.0 \pm 1.8) \times 10^9$
10	+α (43.531)	0 (6.5)	0 (1.0)	$(1.2 \pm 2.3) \times 10^{10}$
11	0 (30)	-α (4.4)	0 (1.0)	$(7.2 \pm 1.9) \times 10^9$
12	0 (30)	+α (8.52)	0 (1.0)	$(8.0 \pm 3.7) \times 10^8$
13	0 (30)	0 (6.5)	-α (0.3)	$(7.2 \pm 2.5) \times 10^8$
14	0 (30)	0 (6.5)	+α (1.7)	$(1.2 \pm 2.6) \times 10^{10}$
15	0 (30)	0 (6.5)	0 (1.0)	$(3.2 \pm 1.3) \times 10^{10}$
16	0 (30)	0 (6.5)	0 (1.0)	$(3.5 \pm 1.7) \times 10^{10}$
17	0 (30)	0 (6.5)	0 (1.0)	$(3.0 \pm 1.1) \times 10^{10}$

Note: X_1 is the lactose concentration (g/L), X_2 is pH, and X_3 denotes inoculum size (g/L)

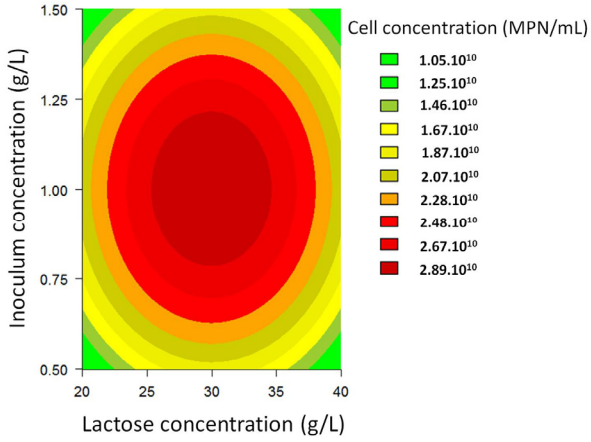

Figure 1: Effect of lactose concentration and inoculum size on the viable cell concentration

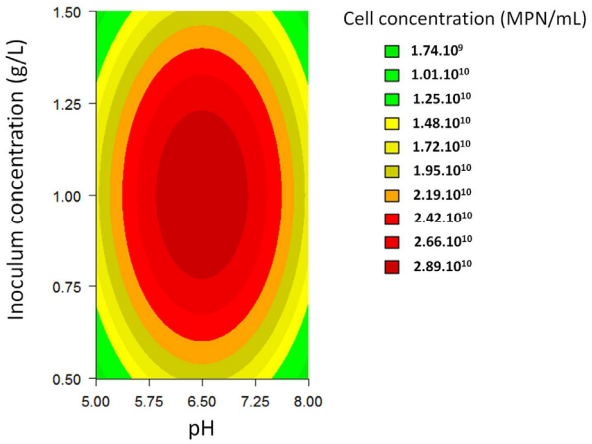

Figure 2: Effect of inoculum size and pH on the viable cell concentration

Growth Pattern in Optimized Conditions

According to the results, diauxic growth patterns were not observed, lactose was hydrolyzed into glucose and galactose and both sugars were utilized simultaneously. Figures 3 and 4 illustrate the dependence of cell, lactose and protein concentrations on the optimized growth conditions. Figure 3 represents the reconstituted whey and Figure 4 the fresh whey, both containing 33 ± 0.5 g/L of lactose. A comparison of the two figures indicates that lactose consumption rates are very similar. According to these figures, the maximum viable cell concentration reached after about 36 h was 10^{10} MNP/mL.

Table 4 list the parameters estimated for the reconstituted and fresh whey fermentation systems. The predictions of the Verhulst model showed good agreement with the experimental results, with a high correlation (R^2>0.99) between the measurements and model predictions. Although the maximum sustainable population in the Verhulst model, X_M, was found to be higher for fresh whey fermentation than for reconstituted whey, the t statistic associated with the estimate of the parameter showed there were no significant differences between the two kinds of medium (Table 5).

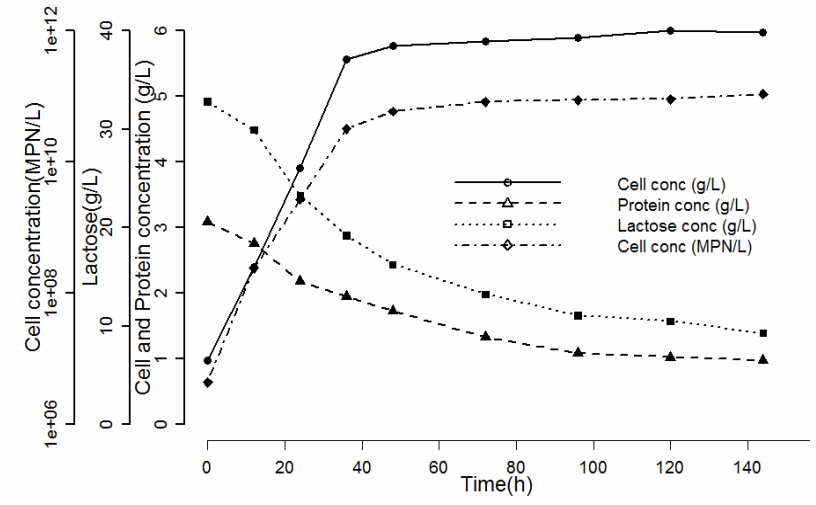

Figure 3: Growth pattern of reconstituted whey fermentation containing 33g/L of lactose under optimized conditions

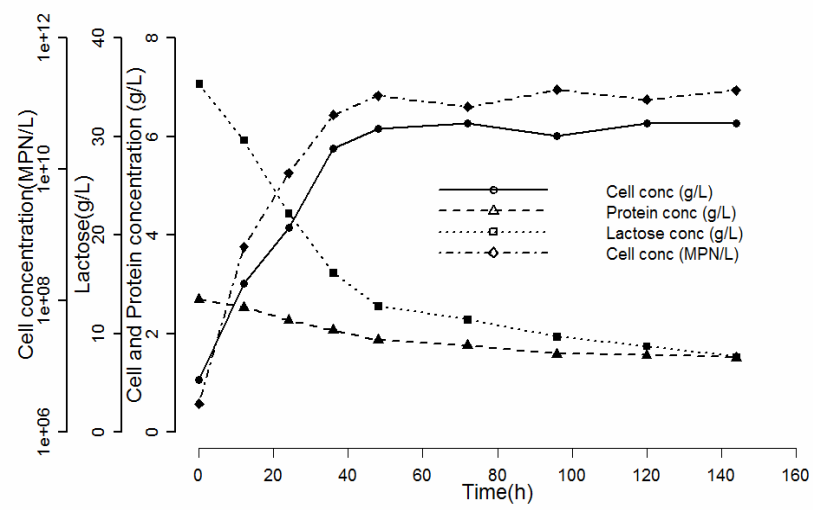

Figure 4: Growth pattern of fresh whey fermentation containing 33g/L of lactose under optimized conditions

Table 4: Fitted parameters obtained for reconstituted whey fermentation

Model	Parameters reconstituted whey (confidence level 95%)		Parameters fresh whey (confidence level 95%)	
Monod	K_S = (72.36 ± 8.40) g/L μ_M = (0.0016 ± 0.0003) h^{-1} $Y_{X/S}$ = (0.0015 ± 0.0002)g/g m = (0.00010 ± 0.00018)h^{-1}	SS = 204.436 R^2 = 0.8655	K_S = (69.99 ± 10.82) g/L μ_M = (0.00450 ± 0.0048) h^{-1} $Y_{X/L}$ = 0.0031 ± 0.0033g/g m = 0.00040 ± 0.000674 h^{-1}	SS = 269.683 R^2 = 0.8079
Verhulst	X_M = (5.65 ± 0.40)g/L μ_M = (0.09468 ± 0.0075) h^{-1} $Y_{X/L}$ = (0.364 ± 0.0432) g/g m = (0.0168 ± 0.0015) h^{-1}	SS = 4.275 R^2 = 0.9958	X_M = (6.26 ± 0.34) g/L μ_M = (0.0902 ± 0.007) h^{-1} $Y_{X/L}$ = (0.215 ± 0.022)g/g m = 0.00398 ± 0.00034 h^{-1}	SS = 3.456 R^2 = 0.9956
Contois	μ_M = (0.179 ± 0.0745) h^{-1} B = (61.70 ± 29.33) g/g $Y_{X/L}$ = (0.218 ± 0.033) g/g m = (0.000301 ± 0.00042) h^{-1}	SS = 65.174 R^2 = 0.9447	μ_M = (0.199 ± 0.0496) h^{-1} B = (59.31 ± 22.65) g/g $Y_{X/L}$ = (0.181 ± 0.042) g/g m = (0.000050 ± 0.00005) h^{-1}	SS = 105.533 R^2 = 0.90864

Note: SS indicates sum of squared deviations

Table 5: Parameter inference for the Verhulst model- statistical difference between the reconstituted whey and fresh whey parameters

Parameter	t-test (reconstituted whey)	t-test (fresh whey)	Significant difference
μ_M	0.00105	0.00105	No*
X_M	0.144	0.131	No*
$Y_{X/L}$	0.0351	0.0351	No*
m	0.00302	0.00302	No*

*Not significant means (95% confidence interval): -2.120≤ t-test value≤ 2.120

Immobilized Cells

The beads prepared by mixing a blend of sodium alginate and harvested cells into a calcium chloride solution were spherical particles with a mean diameter of 2.0 to 2.5 mm containing 10^9 cells/g. After stabilization by drying, the particles presented a final cell concentration of approximately 10^{11} viable cells/g bead (11.53 log10 viable cells/g), with a diameter of 0.76 mm and sphericity of 0.77, as illustrated in Figures 5 and 6. The properties after drying indicated that the cell concentration was about 1.75-fold higher than that reported in the literature (Dembczynski *et al*., 2002), and no undesirable loss of product quality resulting from low cell activity and acute loss of sphericity was observed.

Figure 7 shows the cell survival rate at fixed temperatures of 40, 60 and 70 ºC, while the kinetics and the adjusted R-squared statistic for the Survival Models are presented in Table 6. As can be seen, the activation energies for the predicted death rates are highly consistent with the measured results. The values found indicate good thermal stability and good storage stability (Zhao *et al*., 2008), and are in agreement with the literature. Gomes *et al*. (1998) found activation energies ranging from 37.7 to 58.6 kJ/mol for *A. acidophilus* in reconstituted milk, and Zhao *et al*. (2008) reported an activation energy of 124.43 kJ/mol for encapsulated *Lactobacillus acidophilus* XH1.

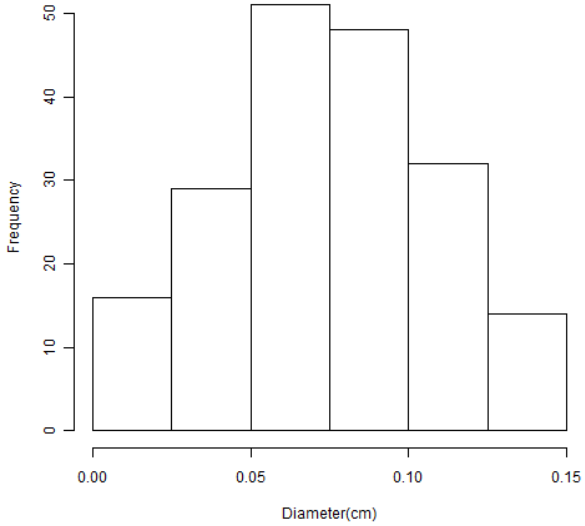

Figure 5: Histogram representation of the particle diameter of dried calcium alginate (mean value of 0.76 mm in a sample of 195 particles).

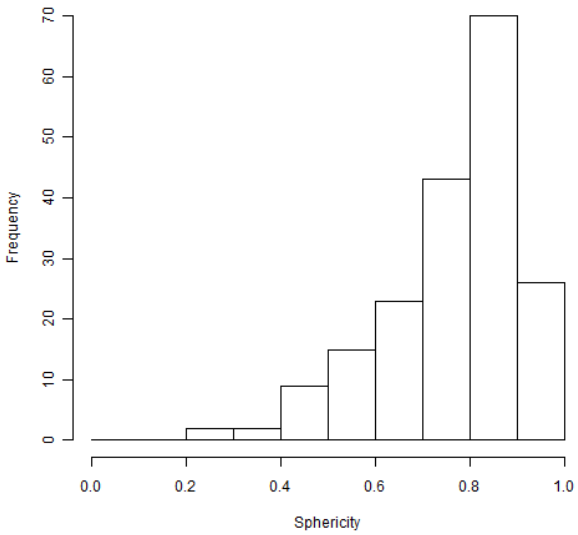

Figure 6: Histogram representation of the particle sphericity of dried calcium alginate (mean value of sphericity 0.77 in a sample of 195 particles).

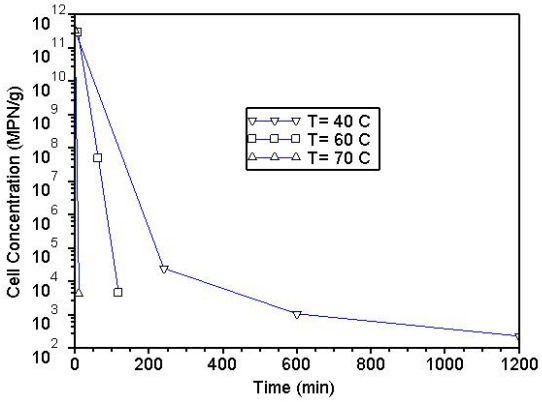

Figure 7: Survival of calcium alginate immobilized cells dried at 40, 60, and 70 ºC.

Table 6: Immobilized cell stability – adjusted R-squared statistic for the Survival Models

Model	Parameters	R^2
First order	Ea = 134.8 kJ/mol $k_0 = 3.83.10^{20}$	0.961
Weibull	Ea = 46.1 kJ/mol $k_0 = 7.75.10^{7}$ n = 0.37	0.998
t^2	Ea = 277.2 kJ/mol $k_0 = 2.18.10^{41}$	0.959

Note: The logistic model revealed no significant parameters

Figure 8 depicts the acid stability tests at pH 2.5 and a temperature of 37 ± 1 ºC for 30, 60 and 90 minutes. The D value found was 62.5 min (R^2 =0.90), indicating that after 6 h of acid deactivation at pH 2.5, the beads remained within the limits of satisfactory viable cells with $5.86.10^5$ viable cells/g.

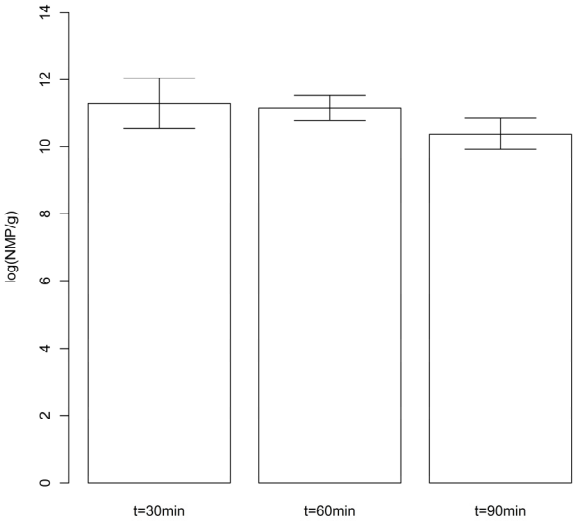

Figure 8: Acid stability tests at pH 2.5 and temperature of 37 ± 1 ºC ($\log_{10}$(N/No)=-t/62.5, R^2=0.90).

CONCLUSIONS

The main conclusions of this study were as follows: 1) the results indicate the potential use of whey to produce immobilized probiotic cells of *Lactobacillus acidophilus* LA 5; 2) The results obtained for reconstituted and fresh whey fermentations showed that there were no significant differences between the two kinds of medium; 3) The factorial design clearly showed that viable cell concentrations are optimized at lactose concentrations of 27 to 35 g/L, an inoculum size of 0.8 to 1.2 g/L, and pH of 6.0 to 7.0; 4) Comparisons of fermentation models for predicting *Lactobacillus acidophilus* probiotic production revealed that the Verhulst model was in good agreement with the experimental results.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. Mônica L. Aguiar of the Laboratory of Environmental Control at the Federal University of São Carlos (UFSCar) for the measurements of particle sphericity and diameter. Financial support from the Brazilian research funding agencies FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) is gratefully acknowledged.

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