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Optimization of fermentation parameters to study the behavior of selected lactic cultures on soy solid state fermentation

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ABSTRACT

The use of solid fermentation substrate (SSF) has been appreciated by the demand for natural and healthy products. Lactic acid bacteria and bifidobacteria play a leading role in the production of novel functional foods and their behavior is practically unknown in these systems. Soy is an excellent substrate for the production of functional foods for their low cost and nutritional value. The aim of this work was to optimize different parameters involved in solid state fermentation (SSF) using selected lactic cultures to improve soybean substrate as a possible strategy for the elaboration of new soy food with enhanced functional and nutritional properties. Soy flour and selected lactic cultures were used under different conditions to optimize the soy SSF. The measured responses were bacterial growth, free amino acids and β-glucosidase activity, which were analyzed by applying response surface methodology. Based on the proposed statistical model, different fermentation conditions were raised by varying the moisture content (50–80%) of the soy substrate and temperature of incubation (31–43 °C). The effect of inoculum amount was also investigated. These studies demonstrated the ability of selected strains (*Lactobacillus paracasei* subsp. *paracasei* and *Bifidobacterium longum*) to grow with strain-dependent behavior on the SSF system. β-Glucosidase activity was evident in both strains and *L. paracasei* subsp. *paracasei* was able to increase the free amino acids at the end of fermentation under assayed conditions. The used statistical model has allowed the optimization of fermentation parameters on soy SSF by selected lactic strains. Besides, the possibility to work with lower initial bacterial amounts to obtain results with significant technological impact was demonstrated.

1. Introduction

Soy is a traditional and central food for Eastern cultures and it has been adopted in the West due to the knowledge and dissemination of its nutritional properties and its potential positive health effects. Argentina ranks third in the world in the production of soybean with an output of 40 million tonnes in 2012 (FAO, 2012) and is the first exporter of soy worldwide, so soy has become an important agricultural commodity in the national economy.

Consumption of soybean has been linked to the prevention of cardiovascular and gastrointestinal diseases, cholesterol reduction, cancer, diabetes and obesity (Gonzalez de Mejia and De Lumen, 2006; Jenkins et al., 2000; Kerckhoffs et al., 2002; Setchell, 1998; Singh et al., 2008; Tikkanen and Adlercreutz, 2000). These health benefits are attributed to the presence of several bioactive compounds which can be separated into protein compounds and non-protein compounds, such as isoflavones. Soy protein has a potential biological value because it is an excellent source of essential amino acids (Kellor, 1974). The hydrolysis of soy protein increases its solubility so the intestinal absorption of protein hydrolysates appears to be more effective than intact proteins (Kong et al., 2008). Different processes have been extensively applied to hydrolyze proteins such as acidic or enzymatic hydrolysis. The latter process can be made with commercial enzymes or enzymes from microorganisms. One of the simplest ways of producing food-grade hydrolyzed proteins is to use lactic acid bacteria (LAB), which are generally recognized as safe and are traditionally used to ferment raw materials of vegetable and animal origin (Aguirre et al., 2008).

On the other hand, isoflavones are phenolic compounds of non-steroid nature with structural similarity to estrogen. Isoflavones responsible for the biological effects are the aglycones which are obtained from the hydrolysis of glycosyl-isoflavones by the enzyme β-glucosidase. In soybean the main isoflavones present are those in glycosyl forms, but little amounts of those in aglycone forms can be present (Wang and Murphy, 1994). Probiotic microorganisms including *Lactobacillus* and *Bifidobacterium* have been known to possess endogenous β-glucosidases which can play an important role in the bioconversion of glycosyl isoflavones during fermentation increasing the amounts of biologically active isoflavones (aglycone form) (Marazza et al., 2009; Otieno et al., 2005).

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LAB play a leading role in the production of novel functional foods. Most of the studies with LAB were developed using submerged fermentation (SmF) and there is a lack of detailed information in the literature about the behavior of LAB on soy solid state fermentation or semi-solid state fermentation (SSF). During this process microorganisms are grown on the surface of solid materials with limited water amounts. Based on the metabolic needs of fermentation microorganisms, the control of water activity, oxygen content, temperature, and pH are the most important SSF parameters (Chen, 2013). SSF is a very interesting technological alternative to obtain new products as it uses waste and/or cheap raw material as substrate. In this sense soy is a great substrate to use for its high nutritional value and low cost.

The aim of this study was to optimize different parameters involved in solid state fermentation (SSF) using selected lactic cultures to improve soybean substrate as a possible strategy for the elaboration of new soy food with enhanced functional and nutritional properties. To optimize soy SSF, we used soy flour and selected lactic cultures under different conditions in order to: a) get the best bacterial growth on solid substrate, b) increase the protein digestibility, and c) obtain higher β-glucosidase activity by application of response surface methodology. The variation of initial bacterial concentration on the behavior of selected strains under previously optimized conditions of soy SSF was also investigated.

2. Materials and methods

2.1. Microorganisms and growth conditions

Lactobacillus (*L.*) *paracasei* subsp. *paracasei* CRL 207 and *Bifidobacterium* (*B.*) *longum* CRL 849 were obtained from the culture collection (CRL) of the Centro de Referencia para Lactobacilos (CERELA). These organisms were selected by their ability to grow on soy substrate using its available carbohydrates and produce β-glucosidase enzyme or hydrolyzed proteins. Before experimental use, cultures were propagated (2%, v/v) twice in MRS medium (De Man et al., 1960) for *Lactobacillus* and incubated at 37 °C for 18 h without agitation. *Bifidobacterium* was grown in MRS supplemented with 1% sucrose, 0.00005% vitamin K and 0.0005% hemin, and incubated at 37 °C for 18 h in microaerophilic conditions without agitation. All solutions were sterilized separately (0.22 μm filtration), and then added to the MRS base. In order to obtain the inoculums for the fermentation process, cells at the end of the exponential phase of growth (5.0 mL initial volume) were collected by centrifugation (10,000 g, 10 min, 4 °C), washed twice with sterile physiological solution and resuspended in 2.5 mL of the same solution. This concentration of the starter cultures was important to avoid the change in the initial moisture of the soy pastes.

2.2. Factorial design and solid state fermentation

Response surface methodology was applied to analyze the solid state fermentation parameters with *L. paracasei* subsp. *paracasei* CRL 207 and *B. longum* CRL 849 in terms of growth (*y1*), free amino acids (*y2*) and β-glucosidase activity (*y3*). The response function *y1* was expressed as the pH difference between fermented soy paste at 24 h and uninoculated soy paste treated in the same way (control), or as the difference between final and initial count cells of fermentation for each condition. The response function *y2* was defined as the difference between the amino acid amounts from fermented soy paste at 24 h and control, and *y3* as the difference in the β-glucosidase activity between the fermented paste at 24 h and control. The coded independent variables and uncoded variables are shown in Table 1 with their variation levels.

In order to evaluate the effects of temperature (T) and moisture (M) and their possible interactions on the three responses, the levels of the independent variables were defined according to 2^2 full-factorial Central Composite Design (CCD), comprising 11 experimental runs in 2 blocks: block 1 was conducted with 6 random assays (4 factorial points and 2 central points), and block 2 with 5 random assays (4 axial points and 1 central point) (Tables 2 and 3).

Table 1
Independent variables and levels of variation in Central Composite Design (CCD).

Independent variables	Levels of variation				
	−1.5	−1	0	+1	+1.5
x1: moisture (%)	50	55	65	75	80
x2: temperature incubation (°C)	31	33	37	41	43

The observations on the CCD were fitted to the second-order polynomial model as follows:

$$Y = \beta_0 + \beta_j + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{12} X_1 X_2 + e$$

where Y is the predicted response of the dependent variable, β_0 is the mean of the total observations (constant), β_j is the estimated coefficient of the block for the response surface model, X_1 and X_2 are coded variables and β is the estimated coefficient for each term of the response surface model.

For each assay, SSF employed 150 g of soy paste (wet weight) which was prepared from commercial soy flour and distilled water to achieve the different moisture contents (*x1*), into 250 mL Erlenmeyer. After water was added, the pastes were homogenized and sterilized by autoclaving at 118 °C for 20 min. When the pastes reached room temperature, they were inoculated with 4% (v/w) of each culture, homogenized and uniformly distributed into Petri plates and incubated at the established temperatures for the design (*x2*) during 24 h. Uninoculated soy paste treated in the same way was used as a control. Samples at different times (0, 4, 8, 12, 16 and 24 h) were taken and linear effects such as the quadratic of the proposed variables (M and T) on three responses (*y1*, *y2* and *y3*) were analyzed. The response functions (*y1*, *y2* and *y3*) were used to perform regression analyses and analyses of variance (ANOVA) for the regression. The experiment results were analyzed using the minitab-15 statistical package (MINITAB Inc., PA, USA), and response surface curves were drawn.

2.3. Moisture determination

The initial moisture of the soy pastes (after sterilization) was expressed as wet basis moisture content, experimentally determined by the method 950.46.B AOAC (1995) and calculated according to the

Table 2
Experimental conditions and results of the statistical experimental design for *B. longum* CRL 849.

Assays	Independent variables coded and uncoded		Response functions		
	x1	*x2*	*y1*	*y2*	*y3*
1	75 (+1)	41 (+1)	−1.33	−0.89	16.210
2	65 (0)	37 (0)	−1.62	−4.64	19.182
3	55 (−1)	33 (−1)	−0.81	0.46	27.356
4	75 (+1)	33 (−1)	−1.42	−0.63	21.256
5	65 (0)	37 (0)	−1.62	−4.31	20.195
6	55 (−1)	41 (+1)	−0.63	−5.25	18.677
7	65 (0)	31 (−1.5)	−0.80	0.61	25.910
8	65 (0)	43 (+1.5)	−0.69	−0.13	27.976
9	65 (0)	37 (0)	−1.39	−4.58	20.276
10	50 (−1.5)	37 (0)	−0.95	−1.34	28.569
11	80 (+1.5)	37 (0)	−1.64	−2.72	16.887

Note: *x1*: moisture (%), *x2*: temperature (°C). Response functions *y1*: ΔpH, *y2*: ΔOPA and *y3*: β-glucosidase activity.

Table 3
Experimental conditions and results of the statistical experimental design for *L. paracasei* subsp. *paracasei* CRL 207.

Assays	Independent variables coded and uncoded		Response functions		
	x1	*x2*	*y1*	*y2*	*y3*
1	75 (+1)	41 (+1)	1.720	4.230	21.319
2	65 (0)	37 (0)	1.654	7.688	28.648
3	55 (−1)	33 (−1)	1.233	−2.178	12.166
4	75 (+1)	33 (−1)	1.915	4.404	12.547
5	65 (0)	37 (0)	1.716	4.730	24.911
6	55 (−1)	41 (+1)	1.491	10.201	24.694
7	65 (0)	31 (−1.5)	1.562	0.079	18.700
8	65 (0)	43 (+1.5)	1.386	−1.755	17.360
9	65 (0)	37 (0)	2.164	3.507	24.660
10	50 (−1.5)	37 (0)	1.662	2.069	19.140
11	80 (+1.5)	37 (0)	1.692	1.752	13.790

Note: *x1*: moisture (%) and *x2*: temperature (°C). Response functions *y1*: Δlog CFU/g, *y2*: ΔOPA and *y3*: β-glucosidase activity.

following equation:

$$M_{wb} = \frac{m_{H_2O}}{m_{H_2O} + m_{ds}} \times 100$$

(Zimbardi et al., 2013) where M_{wb} (%) = wet-basis moisture content; m_{H_20} = mass of moisture (g), and m_{ds} = mass of dry substrate (g).

2.4. pH measurements

Changes in pH were monitored during the fermentation of soy paste at 0, 4, 8, 12, 16 and 24 h using a pH meter (SARTORIUS PT-10, Germany).

2.5. Microbial counts

Cell viability was determined by the plate dilution method using MRS agar for *L. paracasei* subsp. *paracasei* CRL 207 and Reinforced Clostridium Medium (RCM) (Hirsch and Grinsted, 1954) in microaerophilic conditions for *B. longum* CRL 849. Serial dilutions of each fermented soy-paste sample were plated in duplicate and the plates were incubated at 37 °C for 48–72 h. The results were expressed as colony forming units per gram (CFU/g).

The cell morphology was examined using a light microscope (CX, Olympus, Tokyo, Japan) using oil immersion (×100). Plate count (CFU/g) and pH were used to study the bacterial growth.

2.6. Free amino acids in soy pastes

The method of *o*-phthaldialdehyde (OPA) was used to determine the amount of free amino acids in fermented and uninoculated soy pastes (control) (Church et al., 1983). The α-amino groups were released by hydrolysis of proteins reactive with OPA in the presence of 2-mercaptoethanol to form a compound that strongly absorbs at 340 nm.

The proteolytic activity was arbitrarily expressed as μmol of glutamic acid (GLu) released by a gram of soy paste, using a standard curve of glutamic acid (BDH Chemicals Ltd., Poole, UK). This amino acid was used as standard because it is present at higher proportion relative to other amino acids in soy protein.

2.7. β-Glucosidase activity

To evaluate the β-glucosidase activity in the fermented soy pastes and uninoculated soy pastes (control), 0.5 g of each paste was mixed with 1.0 mL of buffer McIlvaine pH 6.40 (0.1 M citric acid–0.2 M Na_2HPO_4; McIlvaine, 1921) and homogenized by Vortex. 150 μL of mix acetone/toluene (9:1) was added to permeabilize the cell. β-Glucosidase activity was assessed by measuring absorbance at 405 nm of *p*-nitrophenol (*p*NP) which was released by the action of the enzyme on a specific substrate (*p*-nitrophenyl-β-D-glucopyranoside, *p*NPGlu) (Sigma) (Garro et al., 2006). Briefly, 45 μL of the enzyme extract was mixed with 15 μL of 10 mM *p*NPGlu incubated for 30 min at 42 °C. The reaction was stopped by adding 900 μL 0.25 M Na_2CO_3. One enzyme unit (U) was defined as the amount of enzyme required to release 1.0 mmol of *p*NP per mL per min under the conditions tested.

2.8. Effect of inoculum amount

After the optimization of temperature and moisture, the variation of inoculum amounts was investigated. Pastes were made from commercial soy flour and distillated water to obtain the selected moisture; they were sterilized and inoculated with different amounts of initial inoculums of each strain used as starter (1, 2 and 4%, v/w) of cultures of each strain. Soy pastes were incubated at selected temperature for 24 h in adequate conditions for each strain used as inoculum. Samples at different times (0, 4, 8, 12, 16, 24 h) were taken. The measurement of pH and plate counts, proteolytic activity and β-glucosidase activity were analyzed.

3. Results

3.1. Effects of soy SSF parameters with B. longum CRL 849 on: ΔpH, free amino acids and β-glucosidase

From the exploratory model of the CCD for *B. longum* (Table 2), the ANOVA and the regression analysis and the linear and quadratic effects of variables *x1* (moisture content) and *x2* (temperature) were observed for each response (Table 4). Full model coefficients were analyzed for their significance and those that were not significant were removed from the model.

The regression coefficients (Table 4) showed low *p*-values for linear and quadratic effects of moisture and only the quadratic effect of temperature on ΔpH, and the interaction between moisture and temperature was not significant. Further, *p*-value greater than 0.05 indicated that the lack of fit for the model was not significant. The R^2 coefficient was 0.96 confirming the goodness of the model. Thus, the proposed model can be described as follows:

$$Y_1 = -1.579 - 0.416X_1 + 0.300{X_1}^2 + 0.852{X_2}^2.$$

Analyzing the mathematical model (X_1 and X_2 coded variables) and response surface graphs in Fig. 1a and c, it was observed that there is a region in which the drop of pH is higher than −1.5 and it includes moistures more than 65% and temperatures around 37 °C, which is near the central point of the design (coded variables: $x1 = 0, x2 = 0$). For all conditions with low moisture content (50 and 55%) the pH values were higher than 5 (Fig. 1b).

The pH decrease for all assayed conditions is shown in Fig. 1b. The initial pH values of the pastes were 6.30 ± 0.2. The lowest pH was 4.66 at 24 h with 80% of moisture and 37 °C. The highest was 5.53 at 24 h for 65% of moisture and 43 °C. Furthermore for temperatures higher than 40 °C a similar behavior was observed: pH remained practically constant after a determinate time. For example, at 75% and 41 °C the pH drop reached 4.95 at 8 h of growth but, after that, it was constant until 24 h. On the other hand, a gradual reduction of pH up to 24 h with a lower rate of pH drop was observed in low temperatures and low moistures.

Besides, the growth of *B. longum* was affected in extreme conditions of temperature (41 and 43 °C) with viability lost at 12 h of fermentation (data not shown), that is in concordance with the maintenance of pH up to 24 h for these conditions.

Table 4
Analysis of variance (ANOVA) and coefficient values for *y1*, *y2* and *y3* of *B. longum*.

Source	*y1*: ΔpH				*y2*: ΔOPA				*y3*: β-glucosidase			
	df	SS	F	*p*	df	SS	F	*p*	df	SS	F	*p*
Regress	5	1.778	22.48	0.020	5	46.610	6.76	0.028	5	138.606	2.80	0.217
Lineal	2	0.679	21.46	0.040	2	6.071	2.20	0.206	2	93.364	4.72	0.070
Quadratic	2	1.097	34.68	0.001	2	33.105	16.55	0.012	2	41.492	2.12	0.215
R	5	0.079	–	–	5	6.895	–	–	5	49.420	–	–
LF	3	0.030	0.41	0.767	3	6.807	51.21	0.019	3	48.892	61.90	0.016
PE	2	0.049	–	–	2	0.089	–	–	2	0.527	–	–

Effects	*y1*: ΔpH		*y2*: ΔOPA		*y3*: β-glucosidase	
	Coefficient	*p*-Value	Coefficient	*p*-Value	Coefficient	*p*-Value
Intercept	−1.580	0.000	−4.464	0.001	19.880	0.000
Block	−0.001	0.970	−0.105	0.769	−1.286	0.217
T	−0.417	0.284	−1.250	0.093	−1.875	0.299
M	0.078	0.001	0.213	0.739	−4.604	0.036
T ∗ T	0.852	0.000	4.559	0.005	5.290	0.095
M ∗ M	0.300	0.033	2.822	0.063	1.075	0.693
T ∗ M	−0.051	0.735	3.067	0.068	2.044	0.588

Note: ΔpH: $R^2 = 0.96$; ΔOPA: $R^2 = 0.87$; β-glucosidase: $R^2 = 0.77$. R = residual error; LF = lack of fit; PE = pure error; df: degrees of freedom; SS = sum of square.

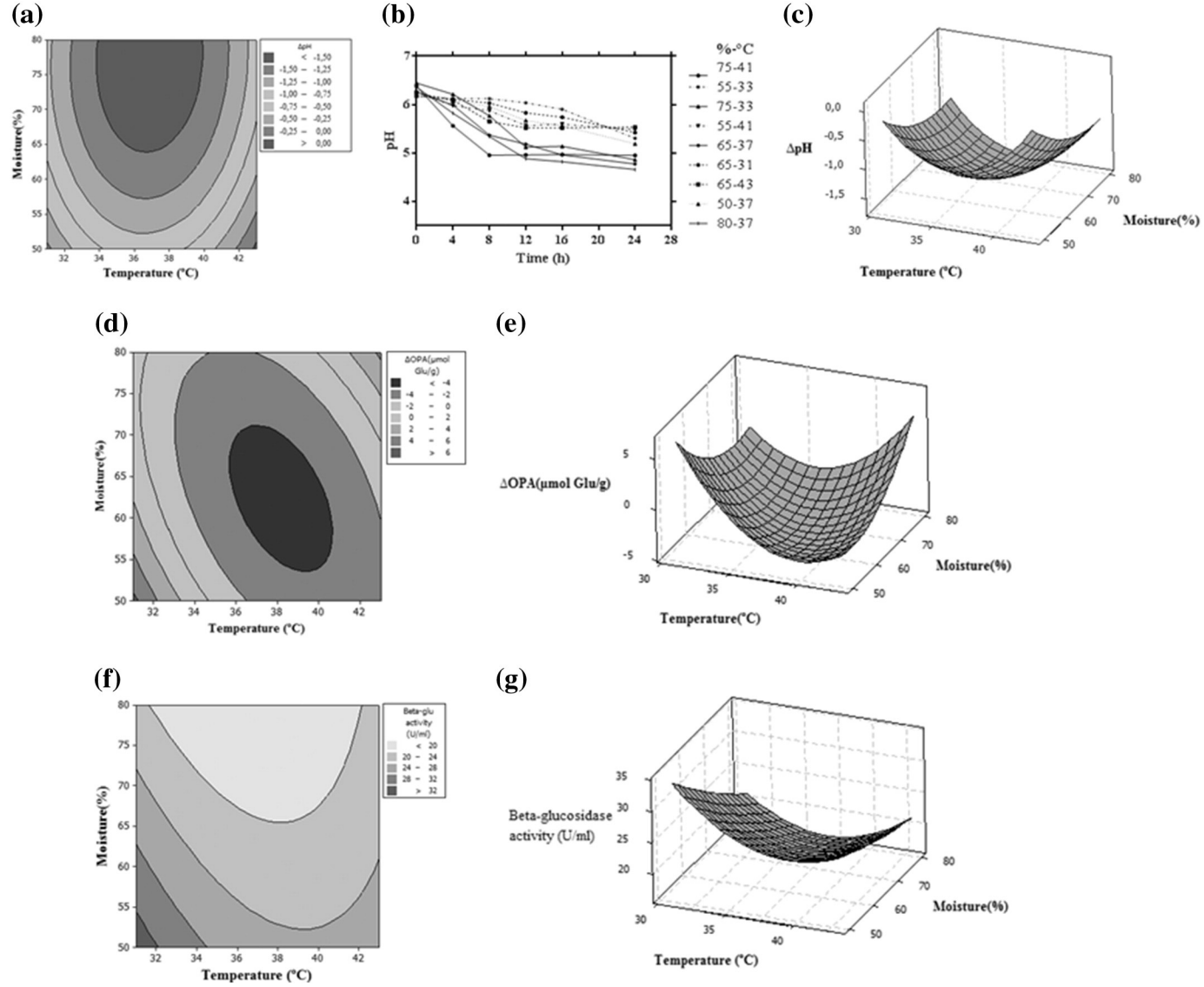

Fig. 1. Response surface graphs for moisture and temperature effects on ΔpH (a and c) and decrease of pH during fermentation times for *B. longum* CRL 849 (b). Response surface plots for *B. longum* CRL 849 showing the interactive effects of initial moisture and temperature on *y2*: ΔOPA (d–e) and *y3*: β-glucosidase activity (f–g).

In order to study the degree of proteolysis, free amino acids at the end of fermentation were measured using the OPA method. The regression coefficients (Table 4) showed low *p*-values for only quadratic effects in both variables and for the interaction between moisture and temperature. The *p*-value for the quadratic effect of temperature was significant ($p \leq 0.05$) on ΔOPA. A depletion of free amino acids at the end of the fermentation with *B. longum* CRL 849 in most tested conditions was observed (Fig. 1d–e).

Furthermore, the β-glucosidase activity was analyzed. The regression coefficients (Table 4) showed only a significant linear effect of moisture ($p \leq 0.005$) and low *p*-values for the quadratic effect of temperature ($p = 0.095$). The interaction between moisture and temperature was not significant. The maximum activity observed was 28.57 U/mL at 50 % of moisture and 37 °C, and the minimums were 16.21 and 16.89 U/mL at 75% at 41 °C, and 80% at 37 °C respectively (Fig. 1f–g).

To choose the optimum conditions for the analyzed responses, the final amount of free amino acids was not considered because ΔOPA was negative. Fig. 2 shows that there is a region in which a great growth and β-glucosidase activity is obtained, and this area is the interception result of each contour graphs and includes the central point of the design. For *B. longum* CRL 849, 65% of moisture and 37 °C were chosen as optimal conditions of fermentation.

3.2. Effects of soy SSF parameters with L. paracasei subsp. paracasei CRL 207 on: Δlog CFU/g, free amino acids and β-glucosidase

For this case, the linear and quadratic effects of moisture and temperature were not significant on any response analyzed ($p \geq 0.05$). In spite of this, surface graphics were drawn to visualize the behavior of this strain for each response.

Analyzing the response surface plots (Fig. 3a and c), it is possible to observe that there is a zone where the Δlog CFU/g is more than 1.8, which includes moisture higher than 60% and temperatures around 37 °C including the central point of the design. This strain was able to grow in all conditions assayed without viability loss, reaching more than 9.5 log CFU/g in most conditions (Fig. 3b). In this case, high temperatures and low moisture have not shown negative effects on growth, but the low moisture content pronouncedly affected pH showing a low reduction or maintaining their values (data not shown).

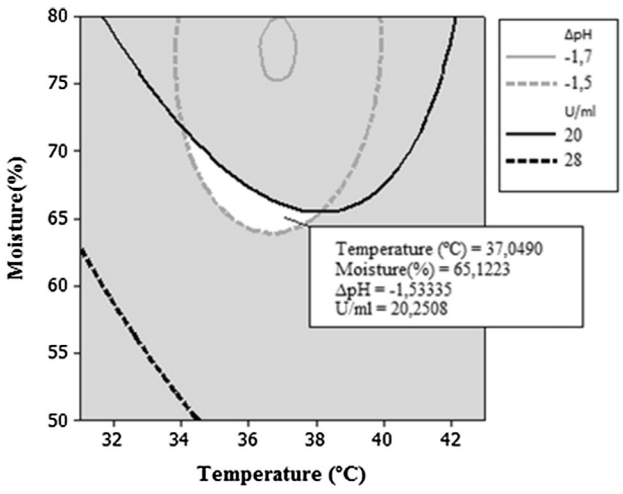

Fig. 2. Superposition of contour graphs of effects of moisture and temperature on β-glucosidase activity and ΔpH. Continuous lines are the minimum value pretended and discontinuous lines are the maximum value pretended. The white zone is the intersection of two contour graphs and it represents the area that includes values of ΔpH and β-glucosidase activity inside the values pretended. White box indicates the values of both responses near to the central point of design.

Besides, this strain showed an increase of free amino acids at the end of the fermentation indicating the possibility of their proteolytic activity. The higher ΔOPA values were 10.2 μmol GLu/g and 5.3 μmol GLu/g for 55% (M) at 41 °C (T), and 65% (M) at 37 °C (T) respectively.

This strain showed a good β-glucosidase activity but the effects of moisture and temperature were not significant ($p \geq 0.05$). Only a low *p*-value for the quadratic effect of moisture was found ($p = 0.073$). Response surface graphs (Fig. 3f–g) show that there is an area where β-glucosidase activity is greater than 25 U/mL and it is near the central point of the design (65% and 37 °C).

The predictions were not carried out because the effects of moisture and temperature were not significant on the analyzed response functions. Despite this, the greater amounts of free amino acids and β-glucosidase activity were observed near the central point of the design. *L. paracasei* subsp. *paracasei* showed an excellent development on all tested fermentation assays. The chosen conditions to work in soy SSF with this strain were 65% of moisture and 37 °C of incubation temperature.

3.3. Analyzing the effect of inoculum amount on the strains' behavior under previously optimized conditions of SSF

The inoculum amounts in SSF with selected lactic cultures were investigated after optimized temperature and moisture. The previous study was developed using 4% of inoculum to secure growth on solid substrate. The decrease of bacterial concentration was analyzed in terms of growth, free amino acids and β-glucosidase activity. No significant differences between inoculums for both strains on the responses analyzed were found.

The *L. paracasei* subsp. *paracasei* CRL 207 population reached around 9.6 log CFU/g for three inoculums at 24 h and the pH decreased slightly in all cases. On the other hand at the end of fermentation the *B. longum* CRL 849 population reached around 9 log CFU/g for three inoculums and pH values descended ca. 1.55 times with respect to control (uninoculated soy paste); nevertheless the acidification rate with 1% inoculum was lower (Fig. 4a–b). CRL 849 showed greater specific growth rate (μ) and acidification than CRL 207 in all cases (Table 5); however CRL 207 showed good development too. Both strains presented β-glucosidase activity at 24 h for all inoculums without significant differences between the three conditions (Fig. 4d). Moreover, the behavior with respect to the free amino acids was maintained consistent with the previous study: an increase of amino acid amount in all cases for CRL 207 and a decrease for CRL 849 (Fig. 4c).

4. Discussion

Interest in soybeans and soy-based products has significantly grown in the last decade due to their reported nutritional and health-promoting benefits. Fermentation with lactic bacteria is a widely used alternative to improve the nutritional and functional value of soy-based products. This work tested the alternative to use selected lactic cultures to increase the protein digestibility and amount of aglycone isoflavones by solid state fermentation in order to enhance nutritionally the soy substrate and obtain a new functional soy-based food. A lot of studies use lactic bacteria to increase the protein digestibility and aglycone isoflavones amount but in most of these cases submerged fermentation was used. Garro et al. (1998, 1999, 2004a, 2004b) in previous studies reported the growth characteristics and the end-product formation of different lactic acid bacteria in soy milk (SM).

Optimization of the fermentation parameters (moisture and temperature) was carried out using response surface methodology (RSM) in the present study. RSM was found to be more satisfactory and effective than other methods due to its efficacy to study many variables simultaneously with a low number of observations, saving time and costs (Bezerra et al., 2008). Moisture and temperature were considered the most relevant parameters to the SSF process (Yadav,

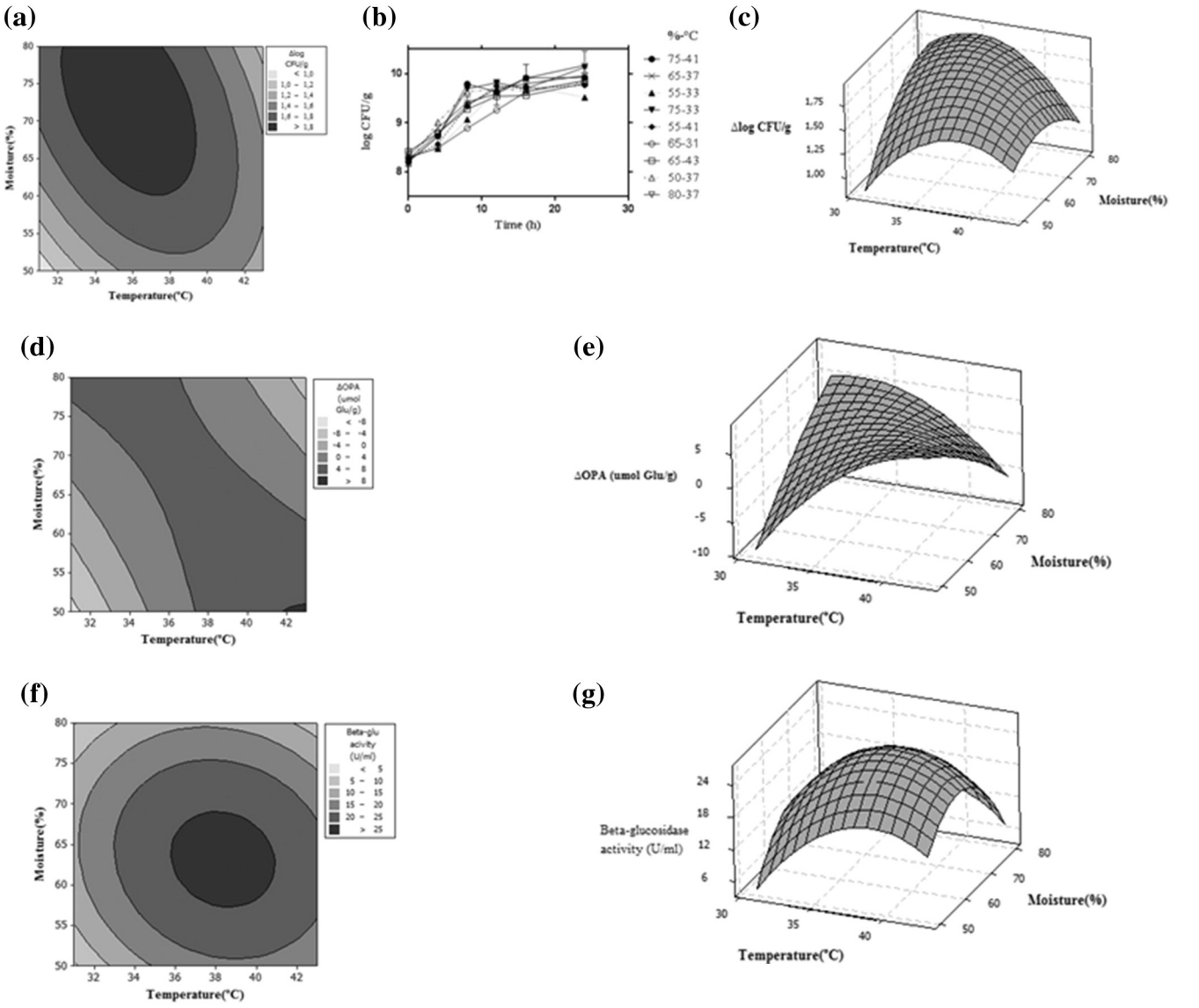

Fig. 3. Response surface graphs for moisture and temperature effects on Δlog CFU/g (a and c) and growth curves for *L. paracasei* subsp. *paracasei* CRL 207 (b). Response surface plots for *L. paracasei* subsp. *paracasei* CRL 207 showing the interactive effects of initial moisture and temperature on *y2*: ΔOPA (d–e) and *y3*: β-glucosidase activity (f–g).

1988) and they were the main parameters associated with the greatest β-glucosidase production in SSF using fungi (Handa et al., 2014). During fermentation, high moisture content decreases porosity and diffusion oxygen, while low moisture content may hinder microorganism growth because of lower nutrient solubility and rapid water loss by evaporation. Further, the incubation temperature influences the growth of microorganisms, product formation and other responses (Lonsane et al., 1985).

This work evaluated the behavior of *L. paracasei* subsp. *paracasei* CRL 207 and *B. longum* CRL 849 on soy solid substrate in terms of growth, free amino acids and β-glucosidase activity in different conditions. Optimization of fermentation parameters allowed obtaining the values of humidity and temperature which not only achieved the desired enzyme activity but also ensured the growth of the microorganism. These strains have a potential probiotic capacity and fermentation conditions to ensure that high viability could be favorable for the future design of the product, so further specific studies should be conducted.

For *B. longum* the great pH drop was achieved at 80% of moisture and 37 °C, and this result was expected because it is known that this bacterium needs high moisture contents for good development. Moreover, we suppose that this major development on SSF was done because when the moisture content is high, the void space is filled with water and the air is driven out, which creates some anaerobiosis (Raghavarao et al., 2003). Bifidobacteria are strictly anaerobic so this condition allows a good growth, with fermentation of the sugar presented in soy flour, and reduction of pH. But for this condition the β-glucosidase activity was one of the lowest with 16.89 U/mL. Hence, 65% of humidity is a core value at which it is sufficient to have a good growth and a great β-glucosidase activity. Besides, this strain showed a pronounced effect of high temperatures, showing that the pH was maintained after 12 h of fermentation and a drop of viability for 41 and 43 °C. The chosen conditions of fermentation were: 65% of moisture and 37 °C of temperature. On the other hand, this strain showed a decrease of free amino acids at 24 h of fermentation. In this way, one possibility is that CRL 849 is a proteolytic bacterium but it is nutritionally exigent and it is possible that the amino acids released from the protein hydrolysis are consumed by the microorganism at the end of the fermentation or it really has no proteolytic activity and it only uses the free amino acids in the substrate to grow. More studies are necessary to evaluate if this strain has proteolytic activity.

Moreover, in spite of the analyses of variance being not significant for most of the regression coefficient for *L. paracasei* subsp. *paracasei* CRL 207 this study represents an interesting approach to know the

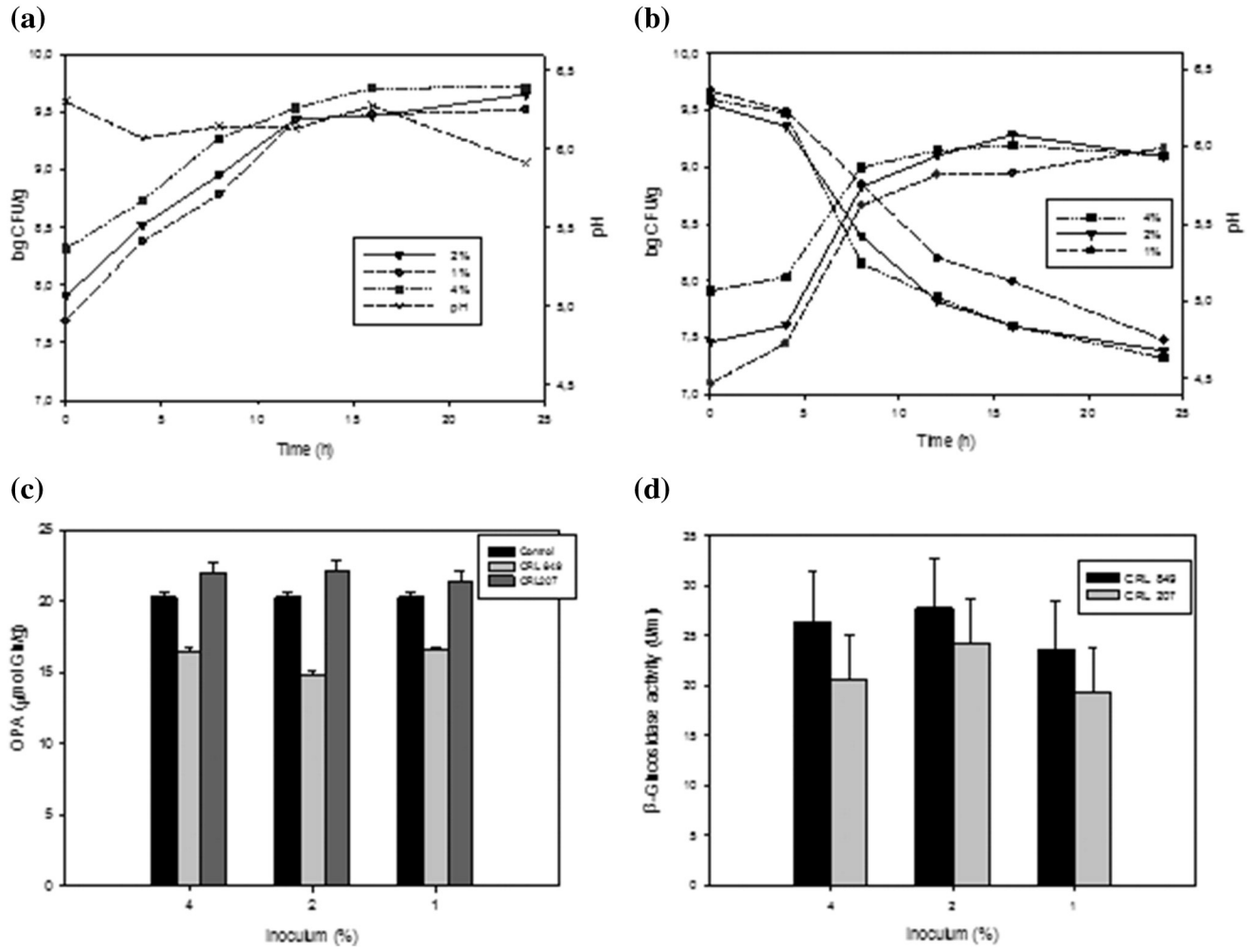

Fig. 4. Growth and pH of *L. paracasei* subsp. *paracasei* CRL 207 (a) and *B. longum* CRL 849 (b) with different initial bacterial concentrations. Free amino acids (c) and β-glucosidase activity (d) of fermented pastes by *L. paracasei* subsp. *paracasei* CRL 207 or *B. longum* CRL 849 with different inoculums. OPA and β-glucosidase activity are the values determined at 24 h of fermentation.

bacterial behavior on this unexplored substrate and give us more information about their development and new horizons to investigate and evaluate the possibility to use SSF to produce new aliments to functional compounds such as more digestible protein and enriched isoflavones. This strain was able to develop in all conditions so it is possible to use it as starter culture in extreme conditions of moisture and temperature (41–43 °C; 50–55% moisture), which represent a technological advantage. Besides, the increase of free amino acids at the end of fermentation could be explained by their proteolytic activity that is in agreement with the finding of Aguirre et al. (2008) where this same strain was used to ferment the SM.

Table 5
Differences in growth (log CFU/g) and pH for *B. longum* CRL 849 and *L. paracasei* subsp. *paracasei* CRL 207 at the end of fermentation (24–0 h) and rate of growth (μ).

	ΔpH	Δlog CFU/g	μ (1/h)
CRL 849 inoculum			
4%	−1.60	1.37	0.320
2%	−1.54	1.83	0.430
1%	−1.52	2.07	0.428
CRL 207 inoculum			
4%	−0.39	1.40	0.232
2%	−0.39	1.75	0.261
1%	−0.30	1.83	0.303

Besides, *L. paracasei* subsp. *paracasei* showed a low acidification on the most tested conditions, and Thi et al. (2003) reported that *L. paracasei* subsp. *paracasei* LG3 is able to grow in a medium called "tofu whey" (TW) where a low acidification was observed, that is in concordance with our work. When other sugar such as glucose was added to the TW, the pH reached lower values. This change on the acidification could be related to the heterofermentative metabolism of this strain, with production of lactic acid, acetic acid and other organic acids when it ferments other kinds of sugar and a higher production of lactic acid when glucose is present in the substrate, with concomitant reduction of pH. But it is possible that not only this heterofermentative metabolism of sugar of *L. paracasei* subsp. *paracasei* CRL 207 was responsible for this behavior on pH values, but also due to its proteolytic activity, and the compounds released from the hydrolysis of proteins are able to buffer the medium. More studies are necessary to understand this behavior.

The three analyzed responses in this work showed the best values near the central point of the design: 65% of moisture and 37 °C. This is in concordance with the optimal variables for *B. longum*. The fact that one of the bacteria is able to increase the free amino acids at 24 h of fermentation and the other bacterium has β-glucosidase activity could represent an alternative to propose the use as co-cultures. Further studies are necessary to optimize growth and to obtain a product more enriched in peptides and bioavailable isoflavones with acceptable technological characteristics.

The possibility to reduce the initial inoculum without modifications on the tested responses was also investigated. No significant differences were found between three inoculums: 1, 2, and 4% (Fig. 4) in terms of growth, free amino acids and β-glucosidase activity at 24 h.

In conclusion this study has given extensive information about the development of *L. paracasei* subsp. *paracasei* and *B. longum* on soy SSF, contributing to the knowledge of lactic starters in such systems. A strain-dependent behavior was observed. The variation of the inoculum for both strains under study was evaluated with no significant differences between the tested inoculums demonstrating that it is possible to work with lower initial concentration of bacterial amounts to obtain results with significant technological impact. The optimal fermentation parameters were: temperature: 37 °C; moisture: 65%; and inoculum: 2%.

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