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IMPROVEMENT OF LACTIC ACID FERMENTATION WITH *Lactobacillus paracasei* ssp. *paracasei* MIUG BL5 ON ENRICHED RYE FLOUR MEDIUM

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The aim of this study was to improve the biotechnological conditions in order to increase the organic acid production with a wild *Lactobacillus paracasei* ssp. *paracasei* strain isolated from pickle juice. The Plackett-Burman Experimental Design (PB), Central Composite Design (CCD) and Response Surface Methodology (RSM) were applied in order to enhance the lactic acid fermentation on a rye flour enriched medium. Among the screened variables, the concentrations of soy protein isolate and KH_2PO_4 as nutrients, time and the temperature of fermentation were identified as the most significant variables that influence the fermented product acidity. The optimized levels of the significant parameters were determined as following: 0.5% soy protein isolate, 0.43% KH_2PO_4, 36 h of fermentation at 31°C, which improved the fermented product acidity approximately with 1.3 folds, compared to the non-optimized fermented product. Furthermore, a good level of cell multiplication rate (10.21 of log N/N0) was achieved.

Keywords: *Lactobacillus paracasei* ssp. *paracasei*, rye flour, lactic acid fermentation, statistical modelling

Introduction

Dough fermentation is one of the oldest food biotechnologies (Rizzelo *et al*., 2016). The product is a mixture of flour (mainly wheat or rye) (Minervini *et al*., 2014) and water that is fermented with lactic acid bacteria (LAB), mainly heterofermentative strains that produce lactic and acetic acid, which are used as natural ingredients for bread making process improvement through stimulation of yeast fermentation and bread preservation. The applications of fermented products in bakery are widely reported, pointing out that these types of products positively influence the organoleptic properties (for instance bread volume, crumb texture and

unique flavour), structural (improved dough machinability), nutritional (through phytate hydrolysis), functional and shelf life properties (De Vuyst and Neysens, 2005; Omedi *et al*., 2016; Rizzelo *et al*., 2016).

In the modern bakery technology, the LAB fermented product represents an alternative to baker's yeast, especially for rye baking (Katina *et al*., 2006), and thus it is used to manufacture a variety of products such as bread, crackers, snacks, pizza and cakes (Corsetti and Settanni, 2007; Aplevicz *et al*., 2013; Minervini *et al*., 2014).

The dough LAB may originate from selected natural contaminants existing in the flour or from a starter culture containing one or more known species of LAB (Vazquez and Murado, 2008). The LAB strains that are part of a typical fermented product mainly belong to the *Lactobacillus* spp. genus and include obligately and facultatively heterofermentative and obligately homofermentative species. However, the obligately heterofermentative lactobacilli, such as *Lactobacillus sanfranciscensis*, have been reported as the most important LAB, especially when it comes to the type I fermented product obtained through continuous daily refreshments at 30°C (De Vuyst and Neysens *et al*., 2005). In general, besides *L. sanfranciscensis*, the dominant LAB species present in the traditional fermented products are *L. brevis* and *L. plantarum*. *L. plantarum* as the part of the fermented product microbiota is not only important for the production of organic acids, but also for several other positive effects expressed during fermentation. *L. plantarum* has been reported as an inhibitor of rope-producing *B. subtilis*, being able to produce bacteriocins, namely plantaricin, and antifungal compounds (Minervini *et al*., 2014; Ventimiglia *et al*., 2015).

Beside the type of the starter culture, in order to improve the fermented product acidity, there are other factors to be taken into account such as the fermentation conditions (pH, temperature and fermentation time) and also the starter's inoculum concentration (De Vuyst and Vancanneyt, 2007).

Statistical experimental methods such as Plackett-Burman design of experiments (PB) and response surface methodology (RSM) had been previously applied to optimize the fermentation parameters (Liong and Shah, 2005; Sawale and Lele, 2010), in order to enhance the acidity of the rye flour fermented product by LAB (Chung *et al*., 2011).

The aim of the research was to investigate the correlative influence of carbon and nitrogen compounds, inoculum, temperature and fermentation time, on the metabolic behaviour of *Lactobacillus paracasei* ssp. *paracasei* used as a starter culture on rye flour based medium in order to improve the acidity and multiplication rate by using the design of experiments and mathematical modelling.

Materials and methods

Chemicals and microorganisms

Man, Rogosa and Sharpe (MRS) broth, agar medium and all other chemicals with high analytical grade were purchased from Sigma Aldrich.

Lactobacillus paracasei ssp. *paracasei* was isolated from pickle juice. The strain was morphologically and biochemically characterized and identified using the MicroStation™ System-Microlog tools (Biolog, USA). The stock culture was stored in 20% glycerol, at -80ºC (Coelho *et al*., 2011).

Culture medium preparation, inoculum and fermentation conditions

A preinoculum was obtained by cultivating the bacteria in a stationary system on MRS broth (De Man *et al*., 1960), through incubation for 24 h, at 30°C. For the fermentation, a basal medium based on rye flour (BM) was used, containing 8.5 g/L reducing sugars. This medium was obtained by mixing 12.5% rye flour with 87.5% tap water, supplemented with 2% malt flour. In order to produce the starch gelatinization, the media was heated up to 75°C and maintained for 30 minutes, followed by a decrease of the temperature until 60°C and maintenance for 5 h. BM was sterilised and then supplemented with carbon, nitrogen and mineral sources according to the optimization design tests. For each sample, 1% of the preinoculum was inoculated into 200 mL of rye flour based liquid medium, with a final optical density (OD) of 0.5, which was measured at the 600 nm, with a UV/VIS V-530 spectrophotometer (Jasco, Japan). Afterwards, the samples were homogenized and incubated in a stationary cultivation system at different times and temperature according to the experimental design.

Optimization of the lactic acid fermentation

Significant variables identification by using Plackett-Burman design

The PB experimental design was used to study the effects of the main factors upon the fermented product (Plackett and Burman, 1946). Dextrose, milk powder, yeast extract, KH_2PO_4, lactose, whey powder, soy protein isolate, inulin, inoculum concentration, temperature and time of fermentation were analyzed as possible factors (independent variables) that influence the lactic acid fermentation. The analyzed responses were titratable acidity and pH. The experimental matrix obtained for the independent variables variation design was described (Table 1). All the variables were designated as numerical factors and investigated in the space intervals -1 (lowest level) and +1 (highest level).

Table 1. Independent variables and levels of variation

Independent variable	Units	Abbr.	Levels of variation	
			-1	+1
Dextrose concentration	g %	A	2.00	6.00
Milk powder concentration	g %	B	1.00	3.00
Yeast extract concentration	g %	C	3.00	5.00
KH_2PO_4 concentration	g %	D	0.10	0.30
Lactose concentration	g %	E	2.00	6.00
Whey powder concentration	g %	F	1.00	3.00
Soy protein isolate concentration	g %	G	0.50	2.00
Inulin concentration	g %	H	0.30	1.00
Temperature	°C	J	25	37
Duration of fermentation	h	K	24	72
Inoculum concentration	$OD_{600\ nm}$	L	0.30	0.70

The effects of the individual parameters on LAB behaviour during fermentation were established based on the following equation:

$$E = \left(\sum M_{+} - \sum M_{-}\right)/N \quad (1)$$

where E is the effect of the studied parameter whereas M_+ and M_- are the responses (titratable acidity and pH) of the trials at which the parameter was at its highest and lowest level, respectively, and N is the total number of trials.

Central Composite Design and Response Surface Methodology

The levels and the interaction effects of the significant parameters were analyzed and optimized by Central Composite Design (CCD). The experimental plan consisted of 27 trials and the independent variables were studied at two different factorial levels (-1, +1), two axial points (α = - 2, α = + 2), and a centre point (0) which is the midpoint of each factor range. All the insignificant variables were numerically optimized in order to increase the organic acids yield (Montgomery, 1997).

The most significant factors, previously determined for PB, such as soy protein isolate concentration (A), KH_2PO_4 concentration (B), temperature (C), duration of fermentation (D), and their variation levels were presented (Table 2). All the experiments were done in triplicate and the average of the total titratable acidity, pH and cells viability were taken into account as dependent variables or responses.

Table 2. CCD of selected independent variables

Variables	Coded levels				
	-α	**-1**	**0**	**+1**	**+α**
Soy protein isolate concentration, A (%)	-0.25	0.50	1.25	2.00	2.75
KH_2PO_4 concentration, B (%)	0.10	0.30	0.50	0.70	0.90
Temperature, C (°C)	19.0	25.0	31.0	37.0	43.0
Duration, D (h)	12.0	24.0	36.0	48.0	60.0

The general form of the second degree polynomial Eq. (2) that was used for the study is the following:

$$Y_i = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j, \quad (2)$$

where Y_i is the predicted response, $x_i x_j$ are the input variables which influence the response variable Y; β_0 is the offset term; β_i is the ith linear coefficient; β_{ii} the ith quadratic coefficient and β_{ij} is the ijth interaction coefficient.

Analytical methods

The samples were taken from each flask (run) in triplicate in order to perform the analytical determinations. Furthermore, the total titratable acidity (TTA), pH and cells multiplication rate were evaluated. The results were expressed as mean of three determinations.

Acidity assay and pH measurement

According to the design of the experiments, the TTA and pH were taken into consideration as being the indicators of the fermented product acidity during the

lactic acid fermentation, at different fermentation intervals. During the fermentation, the potentiometric measurements of pH were carried out with a pin electrode from a MP 2000 pH meter (Mettler Toledo, Switzerland). For the TTA assay, 4 mL of sample was transferred into a 50 mL Erlenmeyer flask and diluted with 46 mL distilled water. After mixing, 10 mL of diluted sample was titrated under shaking with 0.1 N NaOH by using a solution of 1% phenolphthalein (in 70% ethanol) as an indicator, at pH 8.5 Banu *et al*., 2011; Liu *et al*., 2016). The TTA was expressed as the volume (mL) of 0.1 N NaOH used for titration (Iacumin *et al*., 2009; Elhariry *et al*., 2011).

Lactic acid bacteria counting

LAB viable cell number was determined with the plate count method (Liong and Shah, 2005). For this method, serial decimal dilutions with 0.1% (w/v) peptone water (Merck) were prepared and the cells suspension was subsequently plated on MRS agar supplemented with 1% $CaCO_3$, on Petri dishes. The plates were incubated for 48 h, at 30°C. The plates that contained a number of colonies in the range 30–350 were selected for counting and the CFU/mL for the fermented product was calculated. All plate counts were carried out in duplicates. The rate of multiplication was expressed as log N/N_0, where N_0 is the CFU/mL after the starter culture inoculation and N is CFU/mL after 48 hours of lactic acid fermentation.

Statistical analysis

The design of the experiments and the statistical analysis were performed with the Design Expert Software, version 8.0.7.1 (Stat-Ease Inc., Minneapolis, USA).

Results and discussion

Screening of parameters using Plackett-Burman design

When it comes to lactic acid bacteria, from a nutritional point of view, they are fastidious microorganisms that require several amino acids and vitamins to grow (Coelho *et al*., 2011), so it is very important to choose the right nitrogen and carbon sources. In this respect, the refined sugars such as glucose, sucrose, maltose, fructose and lactulose (Coelho *et al*., 2011; Hayek and Ibrahim, 2013) have been used more frequently as carbon source to produce lactic acid than raw starchy substrates, such as barley, corn or wheat. One key driver for the flavour is the carbon source available to the microorganisms, most notably being the low-molecular weight sugars that are flavour precursors (Salim-ur-Rehman *et al*., 2006).

Furthermore, in order to produce lactic acid, a considerable amount of a complex nitrogen source such as yeast extract, peptone, beef extract, skim milk, Soy Peptone Yeast (SPY), whey protein hydrolysate, must be added to the medium within a reasonable timeframe (Hayek and Ibrahim, 2013). It can contribute not only to obtain a high biomass but also a high level of organic acids production through lactic acid fermentation (Liu *et al*., 2010). Some strains of *Lactobacillus* spp. showed a much higher growth rate, cell yield, acidification rate and acetic acid

production when xylose, ribose or arabinose were added to the culture medium, instead of maltose, (Carnevali *et al*., 2007).

The nitrogen source represents a major influence factor on the growth of *Lactobacillus* spp. On the other hand, high concentrations of nitrogen can lead to cell death (Coelho *et al*., 2011). From an industrial point of view, a high number of by-products or wastes had been evaluated as substrates for the lactic acid production in order to decrease the process costs, such as sugarcane, molasses and whey as carbon sources (Coelho *et al*., 2011).

In this study, the first step was to conduct experiments in order to evaluate the effectiveness of the selected independent variables, especially the nutritive sources upon the lactic acid bacteria metabolism. Table 3 presents the results of the correlative effects of the analyzed variables using PB.

Table 3. Plackett-Burman experimental design for the screening of significant independent variables that influence lactic acid fermentation

Run	Independent variables											Responses	
	A	B	C	D	E	F	G	H	J	K	L	TTA, mL NaOH 0.1N	pH
1	6.00	3.00	3.00	0.30	6.00	3.00	0.50	0.30	25.00	72.00	0.30	1.7	4.31
2	2.00	3.00	5.00	0.10	6.00	3.00	2.00	0.30	25.00	24.00	0.70	0.8	5.65
3	6.00	1.00	5.00	0.30	2.00	3.00	2.00	1.00	25.00	24.00	0.30	0.9	5.40
4	2.00	3.00	3.00	0.30	6.00	1.00	2.00	1.00	37.00	24.00	0.30	1.1	4.58
5	2.00	1.00	5.00	0.10	6.00	3.00	0.50	1.00	37.00	72.00	0.30	1.7	4.50
6	2.00	1.00	3.00	0.30	2.00	3.00	2.00	0.30	37.00	72.00	0.70	1.4	4.27
7	6.00	1.00	3.00	0.10	6.00	1.00	2.00	1.00	25.00	72.00	0.70	1.2	4.13
8	6.00	3.00	3.00	0.10	2.00	3.00	0.50	1.00	37.00	24.00	0.70	1.1	4.41
9	6.00	3.00	5.00	0.10	2.00	1.00	2.00	0.30	37.00	72.00	0.30	1.3	4.45
10	2.00	3.00	5.00	0.30	2.00	1.00	0.50	1.00	25.00	72.00	0.70	1.7	4.30
11	6.00	1.00	5.00	0.30	6.00	1.00	0.50	0.30	37.00	24.00	0.70	1.6	4.30
12	2.00	1.00	3.00	0.10	2.00	1.00	0.50	0.30	25.00	24.00	0.30	1.0	4.76

Regression analysis was performed in order to determine the first order polynomial equation that represents the fermented product total titratable acidity (eq. 3) as a function of the independent variables.

$$\mathbf{TTA} = 1.29 + 0.11\ D - 0.18\ G + 0.075\ J + 0.21\ K \quad (3)$$

Statistical analysis of the response showed that the model *F* value was 26.47 which indicated that the model was significant. The values of $P< 0.05$ indicated the fact that the models terms were significant. Among the screened variables, the concentrations of KH_2PO_4 (D), soy protein isolate (G), temperature (J) and duration of fermentation (K) were identified as the most significant variables that influenced the acidity of the fermented product with *Lactobacillus paracasei* ssp. *paracasei*.

In the Pareto chart (Figure 1), it can be observed that the maximal effect on the fermented product acidity was obtained in the superior part of the diagram, followed by a significant decrease, then the minimal effect.

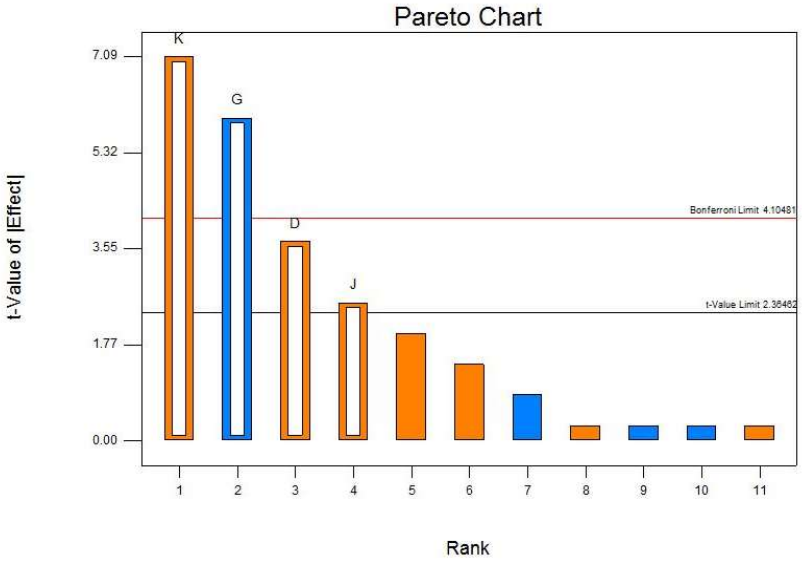


Figure 1. Pareto chart of eleven independent variables effects on the fermented product acidity

Furthermore, it was shown that the most important factors determining the fermented product acidity by *Lactobacillus paracasei* ssp. *paracasei* strain were the concentrations of KH_2PO_4 and soy protein isolate, duration and temperature of fermentation. The data are well correlated with those reported by Juodeikiene *et al*., 2011, who declared that the main factor in acidification is the amount of fermentable carbohydrates, although the production of organic acids depends also on other parameters such as temperature, duration of fermentation and dough yield. Nonetheless, the literature stipulates that many lactobacilli use the soy protein hydrolysate in order to stimulate their growth (Wang *et al*., 2007). Kwon *et al*., 2000 reported that after the optimization process, 15 g/L yeast extract could be successfully replaced with 19.3 g/L Soytone, an enzyme-hydrolysate of soybean meal, supplemented with vitamins, which provided a production of 125 g/L lactic acid from 150 g/L glucose, after the fermentation with *Lactobacillus rhamnosus.*

Decok and Cappelle, 2005 affirmed that besides the fermented product consistency, starter culture and substrate, the temperature during the fermentation is an important parameter that influences significantly the final flavour and the fermented product acidity. On the other hand, when compared to dough yield and acidification rate parameters, the temperature yields a very high influence and furthermore it has also an impact on the microbial composition of the fermented product.

Fermentation process optimization by response surface methodology

CCD and RSM were employed in order to study the interactions between the significant factors (independent variables) and also to determine their optimal levels. The other variables in the study were maintained at a constant level which provided the maximal acidity in the PB evaluation step (Gangadharan *et al*., 2008; Jamal *et al*., 2009). The analyzed responses (dependent variables) were the total titratable acidity, pH and cells multiplication (Table 4).

Table 4. Experimental design and fermentative process optimization results

Run no.	Soy proteins isolate (A), %	KH_2PO_4 (B), %	Temperature (C), °C	Time (C), h	Dependent variables (Response)			
					TTA, mL NaOH 0.1 N		pH	Rate of multiplication, log N/N_0
					Predicted	Actual		
1	0.50	0.30	25.00	24.00	1.13	1.00±0.1	5.33	10.03
2	2.00	0.30	25.00	24.00	1.01	0.70±0.15	5.40	9.98
3	0.50	0.70	25.00	24.00	0.93	1.10±0.05	5.35	10.10
4	2.00	0.70	25.00	24.00	1.23	1.20±0.1	5.30	10.06
5	0.50	0.30	37.00	24.00	1.96	1.70±0.05	4.24	9.96
6	2.00	0.30	37.00	24.00	1.27	1.40±0.15	4.25	10.11
7	0.50	0.70	37.00	24.00	1.93	2.00±0.15	4.26	10.08
8	2.00	0.70	37.00	24.00	1.66	1.80±0.05	4.28	10.09
9	0.50	0.30	25.00	48.00	1.81	1.40±0.05	4.31	9.80
10	2.00	0.30	25.00	48.00	1.77	1.90±0.1	4.33	9.79
11	0.50	0.70	25.00	48.00	1.33	1.40±0.05	4.32	9.74
12	2.00	0.70	25.00	48.00	1.71	1.70±0.05	4.33	9.84
13	0.50	0.30	37.00	48.00	2.47	2.70±0.15	4.13	9.94
14	2.00	0.30	37.00	48.00	1.85	1.40±0.05	4.17	9.99
15	0.50	0.70	37.00	48.00	2.16	2.20±0.05	4.17	9.95
16	2.00	0.70	37.00	48.00	1.97	2.30±0.1	4.20	9.95
17	-0.25	0.50	31.00	36.00	2.02	2.10±0.05	4.19	10.20
18	2.75	0.50	31.00	36.00	1.70	1.70±0.15	4.21	10.53
19	1.25	0.10	31.00	36.00	1.60	2.10±0.05	4.14	10.33
20	1.25	0.90	31.00	36.00	1.52	1.10±0.15	4.20	10.34
21	1.25	0.50	19.00	36.00	1.17	1.40±0.05	5.02	10.19
22	1.25	0.50	43.00	36.00	2.25	2.10±0.15	4.49	10.36
23	1.25	0.50	31.00	12.00	0.92	1.00±0.05	4.93	8.92
24	1.25	0.50	31.00	60.00	1.90	1.90±0.05	4.17	8.30
25	1.25	0.50	31.00	36.00	2.27	2.30±0.1	4.16	10.34
26	1.25	0.50	31.00	36.00	2.27	2.40±0.1	4.15	10.33
27	1.25	0.50	31.00	36.00	2.27	2.10±0.15	4.18	10.35

In order to increase the yield of organic acids based on the PB design, the numerical values for the insignificant variables were optimized as follows: dextrose 4.00 g %, milk powder 1.00 g %, yeast extract 3.00 g %, lactose 2.00 g %, whey powder 3.00 g %, inulin 0.30 g % while the inoculum concentration had an $OD_{600\ nm}$ of 0.50.

Twenty-seven experiments were performed using different combinations of the variables. Thus, based on the experimental results, a second order polynomial

equation (eq. 4) was obtained, which gives the correlation between the fermented product acidity and the soy protein isolates concentration (A, %), the concentration of KH_2PO_4 (B, %), temperature (C, °C) and fermentation duration (D, h) as follows:

$$\mathbf{TTA} = +2.27 - 0.079\,A - 0.021\,B + 0.27C + 0.25\,D + 0.11\,AB - 0.14\,AC + 0.019\,AD + 0.044\,BC - 0.069\,BD - 0.044\,CD - 0.10A^2 - 0.18B^2 - 0.14C^2 - 0.21D^2 \quad (4)$$

The statistical analysis of the analyzed response (TTA) indicates that the quadratic model is significant with a model *F* value of 3.31. There is only a 2.21% chance that a " Model F-Value " this large could occur due to noise. The values of "Prob > F" that are less than 0.050 indicate the fact that the model terms are significant. In this case C, D, B^2, D^2 are significant model terms.

In order to improve the fermented product acidity with *Lactobacillus paracasei* ssp. *paracasei* strain, the temperature and duration of fermentation are to be considered as significant independent variables.

The parity plot showed a satisfactory correlation between the experimental and the predictive values (Figure 2) and since the standard deviation between the experimental and predictive values was very low, the cluster points around the diagonal line indicated the good fit of the model.

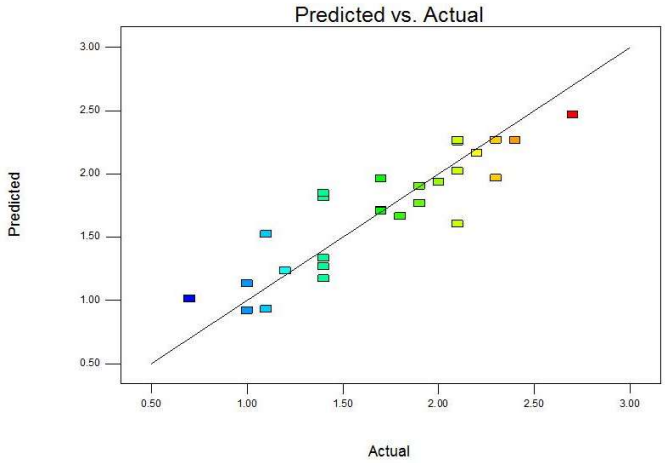
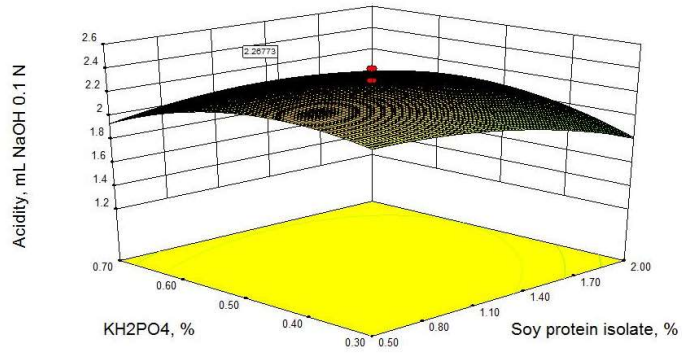


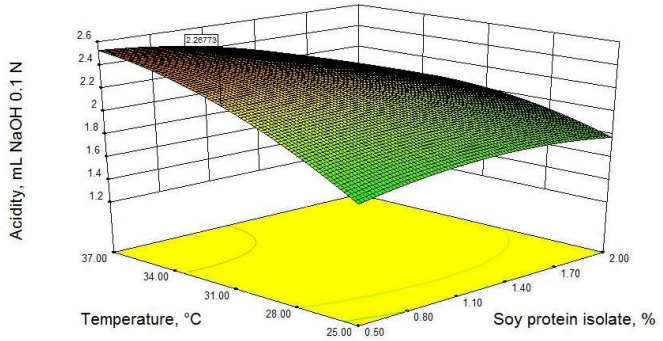
Figure 2. Parity plot showing the distribution of experimental vs. predicted values for the fermented product acidity

Figs. 3 and 4 present the response surface plots for the fermented product titratable acidity with reference to *Lactobacillus paracasei* ssp. *paracasei*. The correlative effect of the soy protein isolate and KH_2PO_4 concentrations with respect to the fermented product acidity improvement is shown in Figure 3A. Thus, the optimized level concerning the fermented product acidity was assessed by maintaining a minimum soy protein isolate concentration (0.50 g%, w/v) and by increasing the KH_2PO_4 concentration up to a central level of variation (0.43 g%,

w/v). In these fermentative conditions the predictive acidity was 2.27 mL of 0.1N NaOH, after 36 h of fermentation, at 31°C.



A)



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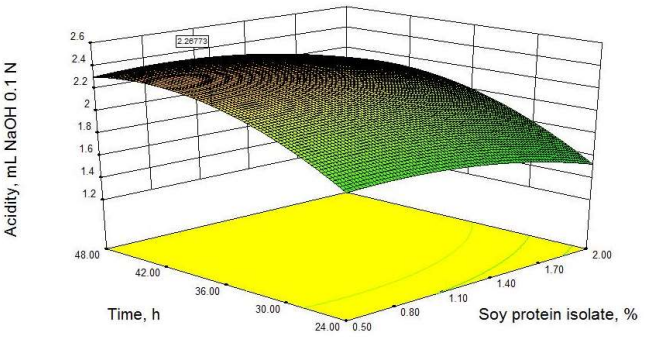
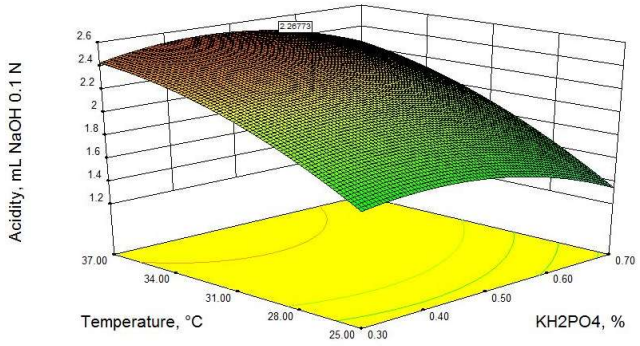


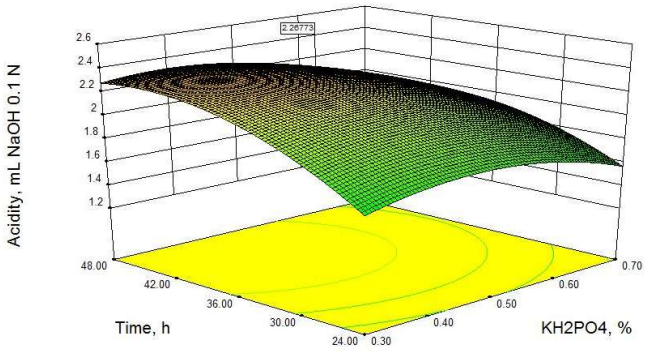
C)

Figure 3. Response surface plot of the effect of soy protein isolate concentration on the fermented product acidity in correlation with: A) KH_2PO_4, B) temperature and C) fermentation time

The interaction between the soy protein isolate, temperature and duration of fermentation (Figs. 3B and 3C) clearly indicates a proper combination for improving the fermented product acidity. In this manner, by increasing the fermentation temperature up to 31°C (Figure 3B) and the duration of fermentation up to the central level of variation (36 h) (Figure 3C), and also by maintaining the concentration of soy protein isolate at a minimum level of variation (0.50 g%), the acidity gradually improved up to 2.27 mL of 0.1N NaOH.



A)



B)

Figure 4. Response surface plot of KH_2PO_4 concentration in correlation with: A) temperature and B) fermentation time

It can be observed that when the concentration of soy protein increases, the fermented product acidity drastically decreases. Also, a comparable interaction effect was observed concerning the increase of the fermented product acidity with *Lactobacillus paracasei* ssp. *paracasei* strain in regards to KH_2PO_4 concentration, time and temperature of fermentation. However, when the concentration of KH_2PO_4 was maintained at a minimum level of variation and the temperature (Figure 4A) and the time of fermentation (up to 36 h) (Figure 4B) increased also, a final acidity of 2.27 mL 0.1N of NaOH was obtained.

In this study, the best results were obtained at 0.3% (w/v) K_2HPO_4, which had a positive effect on the increasement of fermented product acidity with *Lactobacillus paracasei* ssp. *paracasei* strain. The results are in agreement with Coelho *et al*. 2011, which reported that the addition of phosphate to the culture medium increases the lactic acid bacteria growth and enhances the lactic acid production. The highest production of lactic acid was 90.2 g/L, this value being obtained from 220 g/L of molasses, 45 mL/L of CSL, 3 g/L of K_2HPO_4 and 1.5 mL/L of Tween 80. The use of K_2HPO_4 is reported to provide K^+ and phosphate for the microorganism growth and also acts as a buffering agent in the medium (Honorato *et al*., 2007). Furthermore, during *Hericium erinaceus* fermentation, Woraharn *et al*., 2016 suggested that the K_2HPO_4 is the most significant factor, which greatly influences the lactic acid bacteria metabolism during acid production rather than the pH and temperature.

Furthermore, milk powder, K_2HPO_4 and fermentation time were the most significant factors in controlling the fermented product acidity. Hence, a strong interaction between these nutrients in order to increase the fermented product acidity is undeniable. However, at higher concentrations both carbon and nitrogen sources cause the inhibition of lactic acid production due to a significant carbon and nitrogen repression (Coelho *et al*., 2011). According to Honorato *et al*., 2007, the addition of phosphate to the culture medium increases the growth of the microorganism and enhances the lactic acid production, as this component maintains the pH near the optimal growth value, thereby allowing the fermentation to take place for a longer period of time. Kishor *et al*., 2006 reported that when applying the optimum values of the parameters obtained through RSM (25.0 g/L date sugar, 15.0 g/L sodium acetate, 19.1 g/L peptone, and 4.7 g/L K_2HPO_4), the lactic acid production (22.7 g/L) increased by 50.33%, compared to the non-optimized media (15.1 g/L). Instead, according to Kitouni and Oulmi, 2013, K_2HPO_4 as a source of phosphate appears to have no significant effect on the lactic acid production. De Lima *et al*., 2010 reported that the best results for the lactic acid production (52.37 g/L) were obtained with 59.64 g/L of lactose, 14.55 g/L of CSL and 5.65 g/L of $(NH_4)_2SO$, at 39.6°C and pH value of 5.9.

The optimum biotechnological conditions for improving the fermented product acidity under the tested conditions were: 0.50% (w/v) soy protein isolate, 0.43% (w/v) KH_2PO_4, temperature of 31°C, during 36 h of fermentation.

Validation of the model

The validation of the mathematical model was carried out under the predicted conditions by the model, two experiment runs being randomly chosen. The statistical analysis of the obtained data indicated that the titratable acidity values increased over time (2.27 mL 0.1N NaOH, after 36 h of fermentation), the fermentation time and temperature being the most significant variables that influence the acidity of the fermented product with *Lactobacillus paracasei* ssp. *paracasei*. Nonetheless, it was also observed that the lactic acid bacteria presented the highest microbial counts, with a rate of multiplication of 10.21 log N/N_0, after 36 h of fermentation (Table 5).

Table 5. Mathematical model validation

Independent variable				Responses		
Soy protein isolate, g %	**KH_2PO_4, g %**	**Time, h**	**Temperature, °C**	**TTA, mL NaOH 0.1 N**	**pH**	**Rate of multiplication, log N/N_0**
0.50	0.43			2.20	4.30	10.21
0.75	0.30	36.0	31.0	2.20	4.28	10.23
0.90	0.60			2.10	4.34	10.25

These observations are in agreement with those reported by Aplevicz *et al*., 2013. The authors stated that LAB showed the highest counts over time, the highest microbial growth occurring after 10 hours of fermentation, with the highest value for LP2 strain with 8.91 log CFU/g, followed by LP1 strain with 8.66 log CFU/g and SC1 strain with 8.03 log CFU/g. After 6 hours of fermentation, for the LP2 strain it was obtained a value of 8.66 log CFU/g and for LP1 strain 8.33 log CFU/g. Ercolini *et al*., 2013 reported that after 5 days of lactic acid bacteria multiplication, for the rye fermented product, the cell densities presumptively reached values ranging from 9.1 to 9.4 log CFU/g. The maximum fermented product acidity was 2.27 mL 0.1N NaOH, this value being obtained under the optimized biotechnological conditions, with *Lactobacillus paracasei* ssp. *paracasei* strain, after 36 h of lactic acid fermentation in a basal medium. In these conditions, an improvement was observed for the fermented product acidity parameter of approximately 1.3 folds when compared to the values obtained for the non-optimized fermented product.

Conclusions

The statistical approach provided important results for the process parameters optimization so that to enhance the rye fermented product acidity, thus allowing a rapid screening of a large number of variables using *Lactobacillus paracasei* ssp. *paracasei* as starter culture. Soy protein isolate and KH_2PO_4 had a significant influence on the metabolic behaviour of the lactic acid bacteria with a positive effect on the fermented product, and also on the rate of cells multiplication, at the temperature of 31°C and after 36 h of fermentation.

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