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Inoculum concentration and inoculation time for propionic acid production from whey using mixed culture of *Lactobacillus helveticus* and *Propionibacterium freudenreichii* PS-1

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ABSTRACT. The production of propionic acid using skim serum by-products of the dairy industry was investigated, and the influence of inoculum concentration of *Propionibacterium freudenreichii* PS-1 and its time of inoculation for the production of propionic acid was evaluated using mixed culture of *Lactobacillus helveticus* and *Propionibacterium freudenreichii*. Initially, the adjustment of propionic bacteria to higher concentrations of propionic acid was tested and kinetic parameters were determined for growth. A central composite rotational design (CCRD) with 11 experiments was carried out, using different inoculum concentrations of *P. freudenreichii*, which were added to the cultures of lactic bacteria at different times. The maximum specific growth rate for *P. freudenreichii* decreased from 0.11 to 0.03 hour^{-1}and the growth time increased from 32h to 100h when concentration of propionic acid in lactate broth increased to 0.25%. The best result for propionic acid production was 3.78 g L^{-1}, inoculating 5.10^{8} CFU mL^{-1} of propionic bacteria after 6 h. For lactose consumption, the best conditions were inoculum concentration above 5.10^{8} CFU mL^{-1} and inoculation time of *P. freudenreichii* after 6 hours. Therefore, it was demonstrated that skimmed whey can be used as a renewable, low-cost raw material for propionic acid production by mixed culture.

Keywords: skimmed whey, lactic bacteria, propionic fermentation, organic acids.

Concentração de inóculo e tempo de inoculação para produção de ácido propiônico usando cultura de *Lactobacillus helveticus* e *Propionibacterium freudenreichii*

RESUMO. Foi avaliada a influência da concentração do inóculo de *P. freudenreichii* PS-1e do tempo de inoculação para produção do ácido propiônico usando cultura mista de *L. helveticus*e *Propionibacterium freudenreichii* em meio contendo soro lácteo. Inicialmente, foi testada adaptação das bactérias propiônicas a diferentes concentrações de ácido propiônico e foram determinados parâmetros cinéticos de crescimento. Em seguida, foi feito delineamento composto central rotacional (DCCR) com 11 ensaios, usando diversas concentrações de inóculo de *P. freudenreichii* adicionados em diferentes tempos ao meio com bactéria lática. A velocidade específica máxima de crescimento de *P. freudenreichii* diminuiu de 0,11 para 0,03 h^{-1} e o tempo de crescimento aumentou de 32 para 100h aumentando-se a concentração de ácido propiônico em caldo lactato para 0,25% m v^{-1}. O melhor resultado de ácido propiônico foi 3,78 g L^{-1}, usando concentração de bactérias propiônicas de 5.10^{8} UFC mL^{-1}, inoculadas após 4h. Para consumo de lactose, as melhores condições foram concentração de inóculo acima de 5.10^{8} UFC mL^{-1} e tempo de inoculação de *P. freudenreichii* após 6h. Comprovou-se que o soro desnatado pode ser usado como matéria prima renovável e econômica para a produção de ácido propiônico por cultura mista.

Palavras-chave: soro desnatado, bacterias láticas, fermentação propiônica, ácidos orgânicos.

Introduction

Cheese production is an important economic activity in Brazil and reached 1.105 million tons, according to estimates by the Brazilian Association of Cheese Industries (*Associação Brasileira das Indústrias de Queijo* [Abiq], 2015). On average, to produce 1 kg of cheese, 9 L of whey are generated (Prazeres, Carvalho, & Rivas, 2012).

Whey, the main by-product of the dairy industry, has aroused the interest of many researchers around the world because of its nutritional and functional capability (Jankowska, Chwiałkowska, Stodolny, & Oleskowicz-Popiel, 2015). It is composed primarily

of 93-94% water, 6-7% of dry matter, 4.5-6% lactose, 0.6-1.1% protein, 0.06-0.5% fat, 0.8-1.0% minerals, and its biological oxygen demand (BOD) and chemical oxygen demand (COD) values range from 27 to 60 kg m^{-3} and from 50 to 102 kg m^{-3}, respectively (Prazeres et al., 2012). Several studies have been conducted to develop new products in order to use whey, since the problem faced by the dairy industry worldwide is that only part of the whey produced is used, the rest is discarded as effluent because of the high cost and difficulty in processing it (Magalhães et al., 2011), despite its high nutritional value. The dairy industry is looking for alternative uses for cheese whey, as environmental legislation has become stricter. One possibility is its use in the propionic fermentation, wherein lactose and lactate whey can be converted to propionic acid by propionic bacteria (Kośmider, Drożdżyńska, Blaszka, Leja, & Czaczyk, 2010). The propionic bacteria are gram-positive, facultative anaerobic, non-spore forming bacteria that have long been used in the production of Swiss-type cheese and also recently recognized for their probiotic properties for human consumption (Thierry et al., 2011).Therefore, many studies had demonstrated that propionic acid could be efficiently produced through the fermentation with *Propionibacterium* species (Zhu et al., 2012, Wang & Yang, 2013).

Propionic acid is a short-chain organic acid, widely used as an animal nutrition additive and in the manufacture of cellulose-based plastics, herbicides, and perfumes (Zhu et al., 2012). Propionic acid salts are used as preservatives which inhibit the growth of moldin food, and are generally recognized as safe food additives (Coral et al., 2008).

Currently, almost all propionic acid is produced by the chemical synthesis of raw materials derived from petroleum (Tufvesson, Ekman, Sardari, Engdahl, & Tufvesson, 2013). However, manufacturers seek to minimize the use of non-renewable resources (fossil fuels) and maximize the use of renewable raw materials (Kádár, Christensen, Thomsen, & Bjerre, 2011). Therefore, the industry is studying alternative propionic acid production methods using fermentation processes, due to the limitations of non-renewable resources and methods and by the need to preserve the environment (Coral et al., 2008). However, the propionic acid production from fermentation has low productivity due to the propionic acid's inhibition of cell growth and therefore the synthesis of additional propionic acid. Researchers have sought to control the production of cell growth inhibitors and to maximize fermentation productivity. Toward this end, propionic acid-tolerant bacterial strains, reactors with immobilized cells, and reactors with recycling were developed to increase the productivity of fermentation (Feng et al., 2010, Zhu et al., 2012). Another study showed that mixed culture has a great chance of overcoming pure culture fermentations, making it an attractive process for the production of biochemicals compounds (Jankowska et al., 2015).

There are few reports on propionic fermentation using mixed cultures. This research was performed in order to verify the influence of inoculum concentration of *Propionibacterium freudenreichii* PS-1 and the time of inoculation of *P. freudenreichii* PS-1 in the production of propionic acid by mixed culture with *Lactobacillus helveticus.*

Material and methods

Preparation of skimmed whey

Skimmed whey was vacuum filtered with filter paper (8 μm pore and 12.5 cm diameter) to homogenize the raw material and remove suspended solids. The pH of the filtrate was adjusted to 7.0 with 0.2 N NaOH prior to autoclaving. For physicochemical characterization of the serum, aliquots were taken for analysis of titratable acidity, pH (Association of Official Analytical Chemists [AOAC], 2012), and lactose (Antrona method) (Dische, 1962). Analyses were performed in triplicate.

Activation of microorganisms and maintenance of cultures

A lyophilized culture of *Propionibacterium freudenreichii* PS1 was activated in lactate broth according to the methodology of Marcoux, Beaulieu, Champagne, and Goulet (1992), and incubated at 30°C for 48 hours. After incubation, 1 mL aliquots of culture were withdrawn and transferred into test tubes containing 9 mL of peptone water (0.1% m v^{-1}) for serial dilutions. Then, 0.1 mL aliquots of appropriate dilutions were plated on lactate agar and incubated at 30°C for 5 days. Isolated colonies were transferred to agar lactate after confirmation of purity by catalase, morphology, and Gram stain tests. The purified stock culture was activated in lactate broth and incubated at 30°C for 48 hours. After growth, the culture was centrifuged (50,000 rpm 10 min^{-1}) and the supernatant was removed and added to freezing media with glycerol, previously adjusted to pH 7,

and autoclaved at 121ºC for 15 min, according to a methodology adapted from Woskow and Glatz (1991). The cells were frozen at -18ºC in micro centrifuge tubes.

The lyophilized culture of *Lactobacillus helveticus* was activated in test tubes containing 10 mL of Man Rogosa and Sharpe broth (MRS) and incubated at 37°C for 24 hours. The stock culture was made with 12% skim milk and 15% previously autoclaved glycerol, frozen at -18°C, according to the methodology adapted from Woskow and Glatz (1991).

The cell growth of strains of propionic bacteria in increasing concentrations of propionic acid was studied by successive transfers of 100 mL of lactate broth containing 0, 0.1, 0.15, and 0.25% m v^{-1} of propionic acid into the 10% inoculum of the primary culture. The inoculated cultures were incubated at 30ºC and optical density was monitored by reading at regular times the absorbance at 660 nm as a measure of growth. Cultures with 10^8 CFU mL^{-1} were transferred into lactate broth with higher propionic acid concentrations, according to methodology described by Woskow and Glatz (1991) and modified by Zhu et al. (2012). The experiments were performed in triplicate.

Determining microbial growth parameters

Parameters of microbial growth, such as maximum specific growth rate (μ_{max}), growth time (tg), length of the lag phase (tlag), and generation time were determined using the predictive model methodology described by Baranyi and Roberts (1994).This model is widely used in predictive microbiology, applicable to conditions with dynamic variations of the environment and has a good ability to adjust. Growth parameters were obtained by fitting the data to the equation from Baranyi and Roberts, using DMFIT 3.0 software.

Standardization and preparation for inoculum fermentation

For standardization of inocula, the growth of *P. freudenreichii* and *L. helveticus* was accompanied by absorbance and plate count measurements by transferring 10 mL of activated *P. freudenreichii* culture to 100 mL of lactate broth, and regular measurements of the absorbance were taken at 660 nm in a spectrophotometer FEMTO model 700 s. For each reading, serial dilutions in 0.1% (m v^{-1}) peptone water were taken and aliquots of 1 mL were transferred to plates and added to lactate agar, employing the technique of plating in depth. The plates were incubated at 30ºC for 5 days and the colonies were quantified in sequence. The *L. helveticus* growth curve was carried out in MRS broth. Periodic absorbance readings (DO 540 nm) were carried out using aliquots of the culture plated in MRS agar and incubated at 37ºC for 3 days. Colonies were quantified in sequence.

In preparing the inoculum for fermentation, aliquots of 10% (v v^{-1}) activated *P. freudenreichii* culture were transferred to flasks containing 100 mL of sterile lactate broth and incubated at 30ºC for 24 hours. Aliquots of 10% (v v^{-1}) of activated *L. helveticus* culture were transferred to flasks containing 100 mL of MRS broth and incubated at 37°C for 24 hours. After this period the cultures were used. The concentration of cells in the inoculum of the two cultures was determined from the optical density. The inoculum concentration was 10^7 CFU mL^{-1} for *L. helveticus* (Abs_{540} = 0.92) and *P. freudenreichii* PS-1 concentration was defined according to experimental design.

Experimental design

The central composite rotational design (CCRD) was used with the following variables: concentrations of *P. freudenreichii* PS-1inoculum *(x_1),* time of *P. freudenreichii* PS-1 inoculation (x_2), in a fixed inoculum concentration of *L. helveticus*of 10^7 CFU mL^{-1}. Experiments were performed as full factorial 2^2, with 4 factorial scores (± 1 levels) with triplicate at the midpoint level (0) and four axial points (± 1.41), totaling 11 experiments. The response variables were the production of propionic acid (PPA) and acetic acid (PAAC) and the variation of cell growth of *P. freudenreichii* PS-1 and *L. helveticus* factor.

Statistical analysis evaluated the effects of the independent variables, using Statistica v.8.0 software. The range of values studied in the experimental design with the coded and real variables is shown in Table 1, according to the methodology proposed by Rodrigues and Iemma (2014) and Suman, Urbano, Leonel, and Mischan (2011).

Table 1. Coded and real values used in CCRD.

Var.	-1.41	-1	0	1	1.41
X_1	7.0	7.3	8.0	8.7	9.0
X_2	2.6	4.0	6.0	8.0	9.4

[a]X1: Log CFU mL^{-1}; X2: Inoculation time (hour).

The model validation was performed through the repetition of a test in triplicate under conditions for the best consumption of lactose (CL). The predicted concentration of CL was then compared with experimental data.

Fermentation and kinetic study

Fermentations were conducted in 500 mL Erlenmeyer flasks initially containing 300 mL of skimmed whey supplemented with 1% yeast extract. The pH was previously adjusted to pH 7 with 0.2 N NaOH and autoclaved at 121°C for 15 min. The two cultures were prepared as mentioned before (Standardization and preparation for inoculum fermentation) and transferred to flasks containing varying amounts of serum (according to the experimental design) and incubated at 30°C for 168 hours without stirring.

Then, 10 mL sample and 1 mL of fermentation broth were withdrawn after 0, 24, 48, 96, 120, 144, and 168 hours. Ten mL were centrifuged at 3000 rpm (1428 g) for 25 min. The supernatant was collected and transferred to test tubes with threads and frozen for later analysis of propionic acid, acetic acid, lactic acid, acidity, lactose, and pH. Viable cells were quantified by plating in depth.

Cell growth factor (Gf) was determined using Equation 1:

$$Gf = (Cf/Ci) \quad (1)$$

where:

Cf is the lactic acid and propionic bacteria concentration at the final of fermentation, and Ci is the initial lactic acid and propionic bacteria concentration.

Characterization of skimmed whey

The pH values were determined by direct reading of the supernatant via a digital potentiometer (banking model Q400AS). For determination of total acidity, total solid content and ash content were used in accordance with the methods of the (AOAC, 2012).

The fat content of the samples was determined according to the Gerber method. Total nitrogen was measured by the micro-Kjeldahl method, according to the methodology of AOAC, with a conversion factor of 6.35 to obtain the total protein content (AOAC, 2012). Lactose was quantified via adopted Antrona methodology (Dische, 1962).

Determination of organic acids

Organic acids were identified and quantified by HPLC (High Performance Liquid Chromatography) in a Shimadzu chromatograph with a conductivity detector (CDD-6A), polarity +, using a pre-column Shim-Pack SPR-H(G) (50 x 7.8 mm) and two columns in series (Shim-Pack SPR-H (250 x 7.8 mm)). The mobile phase was a mixture of 16 mM Bis-Tris, 4 mM p-toluene sulfonic acid, and 100 µM EDTA, with flow rate of 0.8 mL min^{-1}. The injected sample volume was 20 µL and the injection temperature was 45°C. The samples were centrifuged, filtered (membrane porosity 0.22 µm and diameter 25 mm), and diluted with deionized water before injection into the chromatograph. The acid peaks were identified by retention time, using the retention times of the standards for each acid as a comparison. To quantify, curves were drawn from the peak area against concentration of standard (mg mL^{-1}).

The production parameters in g L^{-1} were determined via Equations 2 and 3 for propionic and acetic acid, respectively, and lactose consumption was determined by Equation 4:

$$PPA = [PA]\ final - [PA]\ initial \quad (2)$$

$$PAC = [AC]\ final - [AC]\ initial \quad (3)$$

$$CL = \frac{(Lac_I - Lac_F) * 100}{Lac_I} \quad (4)$$

In which [PA] - concentration of propionic acid in g L^{-1}; PPA - production of propionic acid in g L^{-1}; [AC] - acetic acid concentration g L^{-1}; PAC production of acetic acid g L^{-1}, [CL] -% consumption of lactose, Lac_I – initial lactose concentration in g L^{-1} and Lac_f – final lactose concentration in g L^{-1}.

Results and discussion

Propionic bacteria growth in increasing concentrations of propionic acid

In Table 2 are shown the growth parameters estimated by the primary models of Baranyi and Roberts regarding the influence of propionic acid concentration are shown. High determination coefficients (R^2) were obtained with the adjustment of this model, such as 0.9867, 0.9830, and 0.94886 for propionic acid concentrations of 0.10, 0.15, and 0.25%, respectively, indicating a good fit of the experimental data to Baranyi and Roberts's model. It can be seen from Table 2 that there was a significant increase in the growth time (from 32 to 100 hour) and the generation time (6,1 to 25,1 hour) between lactate broth without propionic acid and with propionic acid at 0.25% m v^{-1}, respectively.

Table 2. Growth parameters for *P. frendenreichii* in lactate broth with 0.10, 0.15, and 0.25% propionic acid (PA) at 30°C obtained by Baranyi and Roberts model.

Media	T (hour)	μ_{max} ($hour^{-1}$)	t_{lag} (hour)	t_G (hour)	R^2
0% PA	32.0	0.1137	14.0	6.1	0.990
0.10% PA	40.0	0.0679	13.1	10.2	0.987
0.15% PA	55.0	0.0507	23.3	13.7	0.983
0.25% PA	100.0	0.0277	55.5	25.1	0.949

It was also noted that cell growth of propionic bacteria in conditions of 0.25 % m v^{-1} of propionic acid showed a much greater lag phase (t_{lag} = 55,5 hour) and a maximum specific growth rate (μ_{max}) much smaller (0.00277 $hour^{-1}$) than in the other cultures with lower concentrations of propionic acid, especially in cultures with lactate broth alone, which had only 14 hour of lag phase and μ_{max} equal to 0.1137 $hour^{-1}$. Longer lag phase and lower maximum specific growth rate indicate that *P. freudenreichii* PS-1growth is influenced negatively by increasing propionic acid concentrations.

This decrease in specific growth rate with increasing concentrations of propionate was also reported by Nielsen, Martinussen, Flambard, Sørensen, and Otte (2009). However, Woskow and Glatz (1991) investigated *P. acidipropionicii* growth in sodium lactate broth at 32°C, with different propionic acid concentrations. A strain tolerant to propionic acid was developed (*P. acidipropionicii* P200910) with a maximum specific growth rate of 0.16 $hour^{-1}$ in sodium lactate broth containing 1% m v^{-1} propionic acid whith a small decrease in μ_{max} when comparing with growth rate in sodium lactate broth without propionic acid.

Therefore, propionic bacteria suffer inhibition for the increase of propionic acid concentration. Other authors have shown that *P. acidpropionicii* is able to grow at a maximum specific rate of 0.08 $hour^{-1}$ when glucose and lactose are co-metabolized, thereby reducing cell growth (Marcoux et al., 1992), which can be partially related to pH decrease.

The problems in fermentation low productivity are due to various environmental factors and the fermentation process itself. Thus, the propionic bacteria adjustment to increased concentrations of propionic acid may be an alternative to minimize these problems.

Influence of concentration and time inoculation of P. *freundenreichii* PS-1 in lactose consumption and propionic and acetic acids production

Table 3 shows the results of the dependent variables of the Central Composite Rotational Design (CCRD), which are the production of acetic and propionic acids, lactose consumption, and variation of cell growth factor over 168 hour of fermentation.

Table 3. Propionic and acetic acid production, lactose consumption and cell growth factor in CCRD.

Experiment	X1 (Log CFU mL^{-1})	X2 (TI hour)	PPA (g L^{-1})	AAP (g L^{-1})	%CL	Gf
1	-1(7.3)	-1(4.0)	1.35	2.60	48.37	0.02
2	1(8.7)	-1(4.0)	3.78	2.16	49.44	0.38
3	-1(7.3)	1(8.0)	1.75	2.82	56.38	0.03
4	1(8.7)	1(8.0)	2.87	1.97	63.19	0.08
5	-1.41(7.0)	0(6.0)	2.07	1.90	67.01	0.39
6	1.41(9.0)	0(6.0)	1.96	2.27	65.81	0.62
7	0(8.0)	-1.41(2.6)	1.64	2.08	47.07	0.85
8	0(8.0)	1.41(9.4)	1.69	2.12	45.15	0.22
9	0(8.0)	0(6.0)	1.56	3.10	44.91	0.54
10	0(8.0)	0(6.0)	1.65	2.13	50.01	0.18
11	0(8.0)	0(6.0)	1.05	2.00	46.45	0.28

X1 - (log CFU mL^{-1}) concentration of *P. freundenreichii* PS-1; X_2 - time of inoculation *P. freundenreichii* PS-1; TIPB hour - *Propionibacterium* inoculation time in hours; PPA- production of propionic acid; AAP - acetic acid production; CL - consumption of lactose; Gf – cell growth factor.

The production of propionic acid varied from 1.05 (experiment 11) to 3.78 g L^{-1} (experiment 2), whereas the production of acetic acid ranged from 1.90 (experiment 5) to 3.10 g L^{-1} (experiment 9). The percentage of lactose consumption in 168h of fermentation ranged from 45 to 67%, with an average consumption of 53%.The highest consumption of total sugars (63-67%) occurred in experiments 4, 5 and 6, with inoculum concentrations of $5x10^9$, $10^8$and 10^{10} CFU mL^{-1}, respectively (Table 3). The variation of cell growth factor was below 1, indicating that the population of cells decreased during the 168 hours for all treatments.

The results of effects of inoculum concentrations and inoculation time of *P. freudenreichii* PS-1 in production of propionic and acetic acids, lactose consumption and cell growth factor in mixed fermentation with *L. helveticus*are shown in Table 4.

Table 4. Effects of inoculum concentrations and inoculation time of *P. freudenreichii* PS-1 in production of propionic and acetic acids, lactose consumption and cell growth factor in mixed fermentation with *L. helveticus.*

Factors	Regression PPA	Regression AAP	p-value PPA	p-value AAP	Regression CL	Regression Gf	p-value CL	p-value Gf
Mean	1.42	2.41	0.02	0.00	47.13	0.34	0.00	0.10
(1) Log CFU mL^{-1} (L)	0.42	-0.10	0.16	0.62	0.78	0.09	0.66	0.41
Log CFU mL^{-1} (Q)	0.45	-0.09	0.20	0.70	9.20	-0.01	0.01	0.91
(2) time (hour) (L)	-0.06	0.01	0.84	0.95	2.39	-0.15	0.21	0.22
time (hour) (Q)	0.27	-0.08	0.41	0.73	-1.01	-0.00	0.63	1.00
1 by 2 L	-0.33	-0.10	0.41	0.70	1.44	-0.08	0.56	0.63
R^2	0.54	0.12	-	-	0.85	0.38	-	-

X1 - (log CFU mL^{-1}) concentration of *P. freundenreichii* PS-1; X2 - inoculation time of *P. freundenreichii* PS-1; PPA- production of propionic acid and AAP - acetic acid production; CL – consumption of lactose; Gf - Cell growth factor.

It was found that all the terms of the independent variables (linear and quadratic) had no significant effect ($p < 0.05$) on the production of propionic and acetic acids and on the cell growth factor. The determination coefficients (R^2) were low for these variables (0.54, 0.12 and 0.38, respectively). This shows that the production of propionic and acetic acids and the cell growth factor were not affected by the inoculum level of *P. freudenreichii* PS-1 nor by the time of inoculation of *P. freudenreichii* PS-1.

For consumption of lactose, the quadratic term of inoculum concentrations was significant ($p = 0.01$) and the determination coefficient was good (0.85). The analysis of variance (ANOVA) for the regression model of lactose consumption is presented in Table 5.

Table 5. Analysis of variance for consumption of lactose (CL).

Factor	SS	Df	MS	$F_{calculated}$	$F_{tabulated(5\%)}$	P
Regression	616.22	5	123.24	5.73	5.05	0.04
Error	107.55	5	21.51			
Total SS	723.77	10				

[SS]Sum of squares, [df]Degrees of freedom, [MS]Mean square.

It is observed in Table 5 that the regression model for the CL data is significant by the Fisher Test, with the Fcalculated > Ftabulated, by p-value (0.04) and by the determination coefficient. So, the regression model obtained for CL is expressed in Equation 5.

$$CL = 47.13 + 0.78\,x_1 + 9.20x_1^2 + 2.39\,x_2 - 1.01\,x_2^2 + 1.44\,x_1\,x_2 \quad (5)$$

In Figure 1 the contour curve for CL is shown and Figure 2 describe the experimental values and those predicted by the model for CL. Analyzing Figure 1, it was possible to determine the conditions for higher consumption of lactose, which is fermentation medium with inoculum concentration above 8.7 Log CFU mL^{-1} and inoculation time of *P. freudenreichii* PS-1 after 6 hours. It is expected that the higher the cell concentration in the medium, the higher the substrate consumption. Figure 3 shows a good agreement between the observed and predicted values indicating a good fit of the model for CL.

To validate the model, an experiment in triplicate with the same conditions of treatment 6 (9.0 log CFU $mL^{-1}$for inoculum concentration and 6.0 hour for inoculation time) was done and the results were an average CL of 65.28% (exp) and a predicted value of 66.5%, with a relative error of 1.28%. It can be observed that the relative error for CL model was low, indicating that the model was validated.

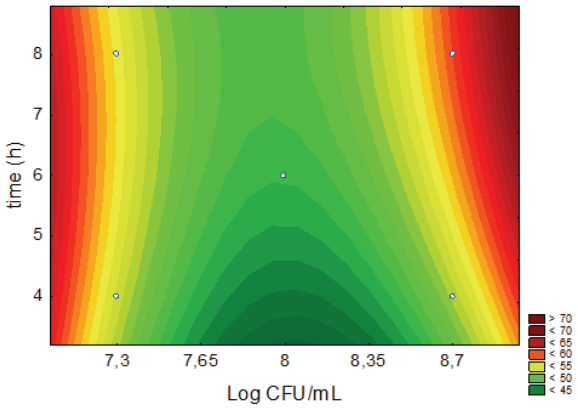

Figure 1. shows the contour curve for CL.

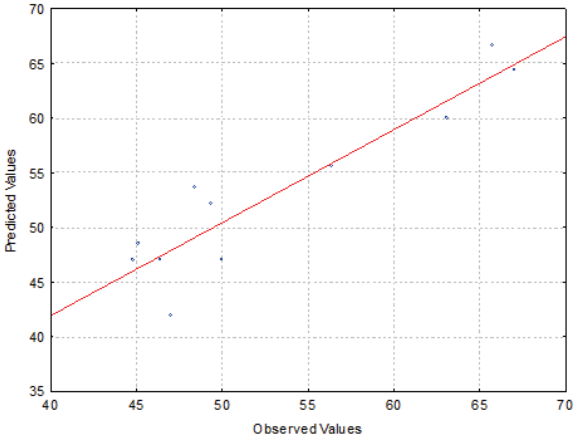

Figure 2. Experimental and predicted values by the model for the CL response.

Acidity and pH

As can be seen in Figure 3, there was a significant increase in acidity up to 96 hour of culture, after this period, and then varied slightly until the end of fermentation for all experiments. Figure 4 shows the pH variation throughout the fermentation for all tests.

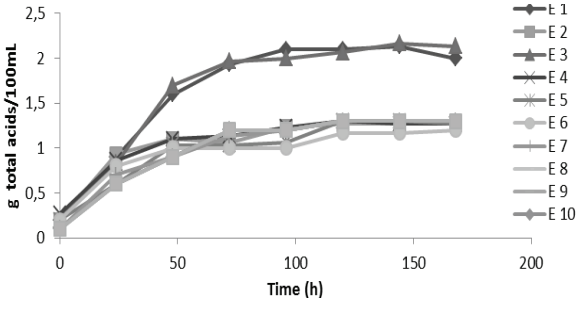

Figure 3. Acidity variation during fermentation in mixed *P. freudenreichii* PS-1 and *Lactobacillus helveticus* culture.

The total acidity in experiments 1 and 3 were higher than the other experiments with the same inoculum concentration of $2x10^8$ CFU mL^{-1}. The

results of titra Table acidity ranged from 0.1 to 1.2 g 100 mL^{-1} for all experiments, except for experiments 1 and 3, whose final acidity reached 2.0 g 100 mL^{-1}. The smallest variations in this study were for tests 4 and 6, with higher inocula concentrations of $5x10^9$ and 10^{10} CFU mL^{-1}. There was no significant difference ($p < 0.05$) in the total acidity of all trials.

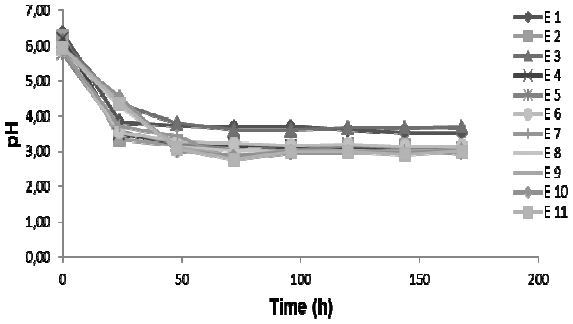

Figure 4. Variation of pH during fermentation in mixed *P. freudenreichii* PS-1 and *Lactobacillus helveticus* culture.

In the first 24 hours of fermentation, the pH values decreased significantly for all experiments, from the mean value of 6.0±0.2 to 3.9±0.4.

Correlating the evolution of acidity with pH, the low production of propionic and acetic acids and variation in cell concentration may be directly related to the low pH values throughout the fermentation, especially after 96 hours. Analyzing the results, it can be observed that the fermentation time was around 120 hours, the same result obtained by Kośmider et al. (2010), that investigated propionic acid production by fermentation at 30ºC with *Propionibacterium freudenreichii* ssp. *Shermanii* utilizing culture media containing 40 g L_{-1} whey lactose and whey lactose with pure glycerol.

There are microorganisms that endure such changes in pH and acidity better than others, but propionic strains of bacteria are not very resistant to this variation. Feng et al. (2010) investigateda pH control strategy for propionic acid production from *Propionibacterium freudenreichii* M207015. It was noted that pH plays a vital role in the production of propionic acid and cell growth; lower pH (5.5) or higher pH (7.0) were not beneficial for propionic acid production and cell growth. This same publication found that pH 6.0 is optimal for the production of propionic acid, having reached maximum production of 14.58 g L^{-1}, but pH 6.5 was optimal for cell growth (Feng et al., 2010). These results are consistent with those obtained in this research.

In this research, propionic bacteria count ranged from an average of $7.51x10^7$ at the start time to $1.83x10^7$ CFU mL^{-1} at the end time. The results of the viable cell count were less than obtained by Marcoux et al. (1992), who used skimmed whey permeate and controlled pH by adding ammonia gas.

The variation in pH and acidity may have favored *L. helveticus,* which can survive at lower pH values. In a study of milk production developed by Nielsen et al. (2009), it was seen that *L. helveticus* are tolerant to pH 3.5, while propionic bacteria can not grow at this pH (Feng et al., 2010). The reduction in cell growth factor can be explained by the low final pH value of the fermentation medium. Thus, it is believed that, in the beginning, there was symbiosis between the two cultures; however, due to the growth of *L. helveticus,* the changes in the fermentation medium, such as acidity, pH and the metabolites produced, may not have favored *P. freudenreichii* PS-1, thereby reducing its cellular growth. This study expected mutual coexistence, as *L. helveticus* ferments lactose, producing lactate which would serve as a substrate for *P. freudenreichii* PS-1 to produce propionic and acetic acids. However, it was noted that this symbiosis was limited due to the increased acidity and pH decrease (Figure 2 and 3) promoted by high lactic acid content. According to Zhu et al. (2012), the concentration of propionic acid may inhibit the bio-production of propionic acid. The pH decrease of fermentation medium may have been the limiting factor in the growth and production of propionic and acetic acids. Although the final concentration of propionic acid (3.78 g L^{-1}) was lower than the best results published in the literature (Feng et al., 2010, Feng et al., 2011, Liu, Zhang, Zhang, Zhang, & Zhu, 2011, Zhu et al., 2012) with the use of other substrates, the results can motivate the use of skimmed whey as a low-cost substrate for the purpose of propionic acid production, using a pH control strategy.

Conclusion

Even at relatively low concentrations of propionic acid in lactate broth (0.25% m v^{-1}), propionic bacteria growth was strongly inhibited by propionic acid, reducing the maximum specific growth rate from 0.1137 to 0.0277 $hour^{-1}$ and increasing the growth time from 32 to 100 hour. The concentration and time of inoculation of *P. freudenreichii* PS-1 did not affect the production of propionic acid, which reached a maximum production of 3.78 g L^{-1}. For lactose consumption, the best conditions were inoculum concentration above 5.10^8 CFU mL^{-1} and inoculation time of *P. freudenreichii* after 6 hour.

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