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Production of Propionic Acid by *Propionibacterium acidipropionici* from Agroindustrial Effluents

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HIGHLIGHTS

- Agroindustrial effluents may be used as substrate for fermentative processes.
- Proposal of propionic acid production using agroindustrial effluents.
- The corn steep liquor and whey were substrates that allowed greater generation of propionic acid.

Abstract: The purpose of this paper was to evaluate the production of propionic acid from the fermentation of agroindustrial effluents using a *Propionibacterium acidipropionici* culture. The composition of the substrates was determined by using an experimental design of mixtures, resulting in 10 trials. The substrates were fermented in batch borosilicate glass reactors at a temperature of 35°C, initial pH of 6.5, and 20 mL.L⁻¹ of inoculum suspension. The highest yield of propionic acid production, 0.79 g of product per g of substrate, was obtained with a substrate composed only of corn steep liquor, which showed a productivity of 5.20 mg.L⁻¹h⁻¹ and production of 0.40mL.L⁻¹. These results showed that the corn steep liquor positively influenced performance and productivity. Although the production of acid did not reach high values, the results indicate that it is possible to produce propionic acid by a biotechnological route; however, further studies are required to adapt and optimise these results.

Keywords: agroindustrial by-products; fermentation; corn steep liquor; cheese whey; wastewater.

INTRODUCTION

Agribusiness generates solid and liquid wastes with high nutritional value. Many of these wastes are not used and, furthermore, treating them to comply with official requirements can be costly, resulting in increases in process costs and, consequently, the final product price. Some companies are already using agroindustrial byproducts, such as corn husks and whey, to produce baked products, enzymes, organic acids and biogas[1,2,3].

There are several by-products that can be used as substrates in fermentative processes. One of these is corn steep liquor, which contains a large amount of nitrogen and amino acids, and is mainly used as a complementary feed in the manufacture of feed for poultry and ruminants [4,5,6]. Whey is a by-product of dairy products that is obtained during the process of milk coagulation. It is generated in large quantities and can cause considerable environmental impact if it is discarded in the environment without proper treatment because it contains high chemical oxygen demand (COD), which causes depletion of oxygen concentration and the death of fish and other aerobic beings [7,8,9]. The effluent from animal feed production consists of residues that originate from the purging of boilers and the washing of floors and equipment during the process of manufacturing animal meal. Its final composition is basically protein but it also contains oils and fats [10,11,12].

Propionic acid is an organic acid used as a preservative in foods and seeds, as well as an ingredient in various products such as thermoplastics, medicines, perfumes, fragrances and solvents. The commercial production of propionic acid is mainly through petrochemical synthesis from ethylene. However, the use of renewable sources to produce this acid by the fermentation process is an environmentally friendly alternative [13,14]. Thus, the objective of this study was to evaluate the production of propionic acid from the fermentation of agroindustrial effluents (whey and corn steep liquor) and effluent from animal feed production using *Propionibacterium acidipropionici* 4843 as inoculum.

MATERIAL AND METHODS

Preparation of the inoculum

The *Propionibacterium acidipropionici* 4843 culture was reactivated in a medium proposed by Parizzi *et al.* [14]. The reactivated strain was transferred to test tubes with screw caps containing 8 mL of storage medium. These tubes were incubated at 30°C for 48 hours. The storage medium for the compound was as follows: 1% glucose; 0.5% yeast extract; 0.5% peptone; 0.1% KH_2PO_4 ; 0.2% $(\text{NH}_4)_2\text{HPO}_4$; and 0.1% of micronutrient solutions 1 and 2. In both media, the pH was adjusted to 7.0 before being autoclaved with 4 mol.L⁻¹ aqueous NaOH solution or 1 mol.L⁻¹ HCl. The micronutrient solution 1 was composed of: 1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.25% $\text{MnSO}_4 \cdot \text{H}_2\text{O}$; 0.5% $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; and 0.5% $\text{FeSO}_2 \cdot 7\text{H}_2\text{O}$. The micronutrient solution 2 was composed of 1% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 1% $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$. The solutions were prepared with Milli-Q water.

Fermentation process

The fermentation process was performed in Duran® borosilicate glass reactors with screw caps and 250 mL volume, which were operated in batch mode. The fermentation took place in a stove, with temperature control of 35°C and without agitation. The pH was monitored every 24 hours during the fermentation process.

Substrates

The substrates used were prepared from a mixture of three types of effluents: corn steep liquor (C); whey (W) and effluent from animal feed production (EF). The effluents were collected and stored in PET (polyethylene terephthalate) polymer bottles and then frozen at -18°C in a freezer until the moment of use. Before being used in the fermentation, they were autoclaved at 121°C, 1 atm for 20 minutes, vacuum filtered in a sterile environment, and stored at 4°C. The composition of the substrates was determined by ternary mixing planning, of the simplex-centroid increased type, and there were 10 trials (Table 1)[15].

Table 1: Three-component simplex-centroid blend planning matrix

ASSAY	CODED VARIABLES			ACTUAL VARIABLES (mL)		
	C	W	EF	C	W	EF
1	1.0	0	0	250	0	0
2	0	1.0	0	0	250	0
3	0	0	1.0	0	0	250
4	0.5	0.5	0	125	125	0
5	0.5	0	0.5	125	0	125
6	0	0.5	0.5	0	125	125
7	0.667	0.167	0.167	166.75	41.75	41.75
8	0.167	0.667	0.167	41.75	166.75	41.75
9	0.167	0.167	0.667	41.75	41.75	166.75
10	0.333	0.333	0.333	83.25	83.25	83.25

* Corn steep liquor (C), whey (W) and the effluent feed production plant (EF).

The pH of the substrates was adjusted to 6.5 with 4 mol.L⁻¹ aqueous NaOH solution or 1 mol L⁻¹ HCl. Prior to inoculation, the media were autoclaved at 121°C, 1 atm for 20 minutes. After sterilization, the media were filtered to remove coagulated material and inoculated with a 20 mL.L⁻¹ suspension of *Propionibacterium acidipropionici* 4843.

Characterisation of the effluents

The composition of the effluents was determined by the analysis of chemical oxygen demand (COD), pH, Kjeldahl total nitrogen (KTN), ammoniac nitrogen (NNH₄⁺), total solids (TS), total fixed solids (TFS) and total volatiles (TV) [16]. The profiles of sugars and organic acids were determined by high-performance liquid chromatography (HPLC).

High-performance liquid chromatography

The determination of sugars and organic acids was performed by high-performance liquid chromatography (Waters 2695 Alliance, Milford MA, USA) using an Aminex HPX-87H

ion exclusion column (300 × 7.8mm) preceded by cationic pre-column Cation-H (Bio-Rad) in an isocratic condition. A 3mM solution of sulfuric acid, prepared in ultrapure water and filtered through a 45µm nylon filter, was used as the eluent. A refractive index (RI) detector was used. The injection volume was 10 µL at a flow rate of 0.6 mL min⁻¹. The column was maintained at 60°C and the refractive index detector was maintained at 35°C. The samples were pre-filtered in a 0.20 µm syringe filter. The chromatographic data were obtained using Empower software.

Productivity and propionic acid yield

The planning response was given by propionic acid yield and volumetric productivity. The sum of glucose, galactose, lactose and lactic acid was considered as the substrate. Formula 1 was used to calculate the yield :

$$Y_{ps} = \frac{(P1-P0)}{(S1-S0)}, \quad (1)$$

Where:

Y_{ps}: propionic acid yield (g of product formed.g of substrate consumed⁻¹);

P0 and P1: initial and final product concentration, respectively (g.L⁻¹);

S0 and S1: initial and final substrate concentration, respectively (g.L⁻¹).

Formula 2 was used to calculate the volumetric yield of propionic acid:

$$Q_p = \frac{(P1-P0)}{(t1-t0)}, \quad (2)$$

Where:

Q_p: volumetric yield of propionic acid (g.L⁻¹.h⁻¹);

P0 and P1: initial and final product concentration, respectively (g.L⁻¹);

t0 and t1: initial and final fermentation time (h).

Statistical analysis

The analyses were performed in triplicate and the results were expressed as mean +/- standard deviation of the mean. The experimental data regarding normality was evaluated using the Shapiro-Wilk test, and homogeneity was assessed by Levene's test. For the data that were considered as normal and homogeneous (p> 0.05), analysis of variance was performed (ANOVA, p-value <0.05 was considered significant) followed by Tukey's test (at 95% level of significance) to compare the media [17]. To evaluate the quantitative effects of the independent variables on the responses of the simplex-centroid experimental design, response surface methodology was applied. The statistical quality was assessed using the coefficient of determination (R²) and adjusted coefficient (R²-adj) [15].

RESULTS

Characterisation of the effluents

The results of the effluent characterisation are presented in Table 2. The effluents show statistically significant differences in relation to the physical-chemical composition.

Table 2. Characterisation of effluents

Parameter (mg.L ⁻¹)	EF	C	W
COD	28239 ± 1039 ^b	19080 ± 1616 ^c	76737 ± 3317 ^a
pH*	5.77 ± 0.06 ^a	4.00 ± 0.10 ^c	5.03 ± 0.06 ^b
N-NH ₄	5255 ± 70 ^a	101 ± 3 ^c	303 ± 7 ^b
KTN	6673 ± 76 ^a	742 ± 25 ^c	1443 ± 32 ^b
TS	4119 ± 55 ^c	15623 ± 230 ^b	46537 ± 745 ^a
FTS	1543 ± 46 ^c	5942 ± 104 ^b	9471 ± 1179 ^a
STV	2575 ± 95 ^c	9680 ± 149 ^b	37066 ± 1639 ^a

* Dimensionless

Different letters in the same line are significantly different according to Tukey's test ($p < 0.05$).

Evaluation of substrate consumption and production of organic acids

Figure 1 shows the behaviour of the pH of the samples in relation to the hydraulic retention time (HRT). It can be seen that the pH declined throughout the fermentation process. In the tests in which higher percentages of whey were present, the final pH had its lowest value, indicating the higher production of fatty acid. Test 3 resulted in the lowest decline, with a final pH of 5.99. Test 4 provided the greatest formation of acids, with a final pH of 4.65. There was a reduction of pH values in all the tests, and it can be concluded that the substrate was consumed, with consequent production of organic acids, in all of the tests, mainly during the first 24 hours of the process.

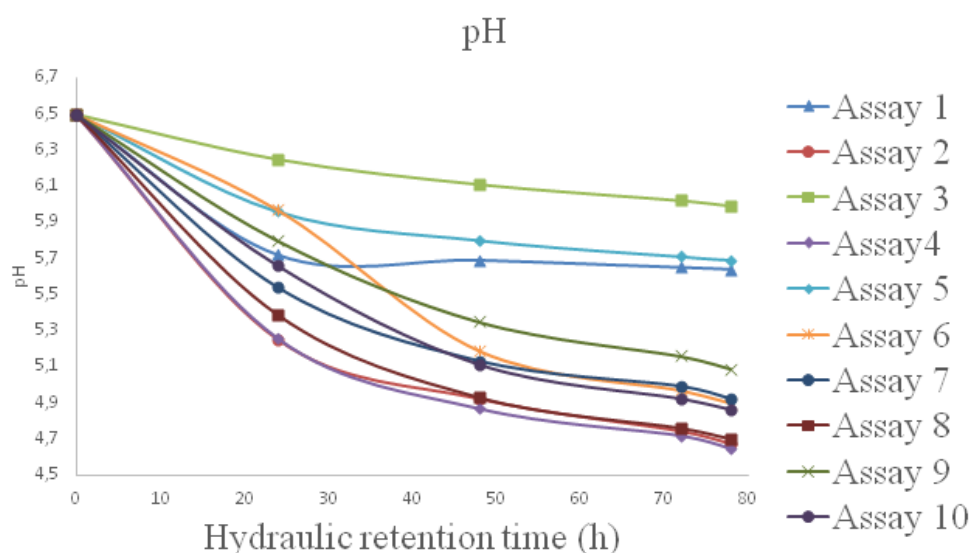


Figure. 1 pH plot over the fermentation time in 10 assays.

The *Propionibacterium acidipropionici* CCT 4843 metabolised the lactose, galactose, glucose and lactic acid present in the studied substrates. The consumption of these nutrients can be seen in Table 3, which shows the amount of nutrients present at the beginning and at the end of the fermentation process.

Table 3. Concentration of nutrients (g.L⁻¹) at the beginning (0h) and at the end (78h) of the trials.

Assay	Lactose		Galactose		Glucose		Lactic Acid	
	0h	78h	0h	78h	0h	78h	0h	78h
1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.51±0.02	0
2	3.54 ± 0.00	3.27±0.01	0.46±0.00	0.17±0.00	0.20±0.00	0	0.30±0.00	0
3	n.d.	n.d.	n.d.	n.d.	0.047±0.00	0.046±0.00	n.d.	n.d.
4	1.68±0.00	1.32±0.00	0.23±0.00	0±0.00	0.08±0.00	0	0.43±0.00	0
5	0.04±0.00	0	n.d.	n.d.	n.d.	n.d.	0.31±0.01	0
6	1.58±0.00	1.52±0.01	0.21±0.00	0.08±0.00	0.07±0.00	0	0.16±0.00	0
7	0.60±0.00	0.36±0.00	0.10±0.00	0	0.05±0.00	0	0.42±0.00	0
8	2.31±0.01	1.92±0.01	0.28±0.00	0.08±0.00	0.13±0.00	0	0.27±0.00	0
9	0.60±0.00	0.41±0.00	0.10±0.00	0	0.05±0.00	0	0.15±0.00	0
10	1.30±0.01	0.71±0.00	0.16±0.00	0	0.07±0.00	0	0.28±0.00	0

*n.d. = not detected. Proportions of the C/W/EF substrates as follows: (1) 1/0/0; (2) 0/1/0; (3) 0/0/1; (4) 0.5/0.5/0; (5) 0.5/0/0.5; (6) 0/0.5/0.5; (7) 0.6/0.2/0.2; (8) 0.2/0.6/0.2; (9) 0.2/0.2/0.6; (10) 0.33/0.33/0.33.

Apart from propionic acid, the organic acids that were detected were acetic and succinic acids, which are products that were generated in the fermentation and can interfere in the production of propionic acid. The amounts of acids generated are shown in Table 4. Table 4 shows that succinic acid was produced in trials 6, 7, 9 and 10.

Table 4. Production of acetic, succinic and propionic acids in fermentation.

Assay	Acetic acid (g.L ⁻¹)	Succinic acid (g.L ⁻¹)	Propionic acid (g.L ⁻¹)
1	0.17±0.01 ^a	n.p.	0.40±0.01 ^{bc}
2	0.11±0.00 ^{bc}	n.p.	0.33±0.01 ^d
3	0.00±0.00 ^f	n.p.	0.00±0.00 ^h
4	0.14±0.00 ^{ab}	n.p.	0.51±0.01 ^a
5	0.03±0.00 ^{ef}	n.p.	0.13±0.01 ^g
6	0.08±0.00 ^d	0.02±0.01	0.28±0.01 ^e
7	0.16±0.01 ^a	0.03±0.00	0.41±0.01 ^c
8	0.14±0.00 ^a	n.p.	0.43±0.01 ^b
9	0.09±0.00 ^{cd}	0.02±0.00	0.23±0.01 ^f
10	0.04±0.01 ^e	0.02±0.00	0.23±0.01 ^f
p-ANOVA**	p<0.001	-	p<0.001

*n.p. = not produced. ** probability values obtained according to single-factor ANOVA. *** Different letters in the same column represent significant difference according to Tukey's test (p <0.05). Proportions of the C/W/EF substrates as follows: (1) 1/0/0; (2) 0/1/0; (3) 0/0/1; (4) 0.5/0.5/0; (5) 0.5/0/0.5; (6) 0/0.5/0.5; (7) 0.6/0.2/0.2; (8) 0.2/0.6/0.2; (9) 0.2/0.2/0.6; (10) 0.33/0.33/0.33.

The yield and volumetric productivity of propionic acid were the parameters used to analyze the performance of the fermentation. The yield indicated the efficiency of the conversion of the substrate into product, i.e, how much product was generated from a certain amount of substrate. The volumetric productivity represents the speed of production; it is an important factor in evaluating the economic viability of the process. The results in

Table 5 show that the yields obtained in the tests ranged from 0.79 to 0.13 g product.g substrate⁻¹; in terms of volumetric productivity the values were between 6.49 and 0.00 mg.L⁻¹.h⁻¹.

Table 5. Productivity and yield for propionic acid.

Assay	Productivity (mg.L ⁻¹ .h ⁻¹)	Yield (g.g ⁻¹)
1	5.20±0.001c	0.79±0.031a
2	4.27±0.013d	0.32±0.004f
3	0.00±0.000h	0.13±0.000h
4	6.49±0.005a	0.46±0.002cd
5	1.71±0.003g	0.38±0.015e
6	3.58±0.009e	0.66±0.014b
7	5.28±0.076c	0.50±0.013c
8	5.52±0.045b	0.43±0.011d
9	2.90±0.141f	0.47±0.030cd
10	2.98±0.014f	0.21±0.003g
p-ANOVA*	p<0.01	p<0.01

*Probability values obtained according to single-factor ANOVA. **Different letters in the same column represent significant difference according to Tukey's test (p <0.05). Proportions of the C/W/EF substrates as follows: (1) 1/0/0; (2) 0/1/0; (3) 0/0/1; (4) 0.5/0.5/0; (5) 0.5/0/0.5; (6) 0/0.5/0.5; (7) 0.6/0.2/0.2; (8) 0.2/0.6/0.2; (9) 0.2/0.2/0.6; (10) 0.33/0.33/0.33.

Tables 6 and 7 show that the presence of corn steep liquor positively influenced the yield, in other words it is a suitable substrate for *Propionibacterium acidipropionici* CCT 4843 to produce propionic acid. The interaction between the substrates was not statistically interesting (negative and non-significant effects) and better results were obtained when there was no interaction.

Table 6 - Estimates of the effects of each factor for RMS in relation to yield.

	Effects	Standard error	t-value	p-value
Corn steep liquor (A)	0.8405	0.1024	8.2077	0.0038
Whey (B)	0.3159	0.1024	3.0845	0.0539
Effluent from animal feed production (C)	0.1642	0.1024	1.6004	0.2070
AB	-0.4865	0.5154	-0.9438	0.4149
AC	-0.3504	0.5154	-0.6797	0.5454
BC	1.8292	0.5154	3.5485	0.0381
ABC	-6.5326	3.3984	-1.9222	0.1503
R ²	0.9125			
R ² adj	0.7376			

Table 7 - Estimates of the effects of each factor for RMS in relation to productivity.

	Effects	Standard error	t-value	p-value
Corn steep liquor (A)	5.2825	0.8841	5.9747	0.0094
Whey (B)	4.3353	0.8841	4.9034	0.0162
Effluent from animal feed production (C)	0.1771	0.8841	0.2003	0.8540
AB	7.3548	4.4505	1.6525	0.1970
AC	-3.0184	4.4505	-0.6782	0.5463
BC	6.2755	4.4505	1.4101	0.2533
ABC	-12.976	29.3409	-0.4403	0.6895
R ²	0.9280			
R ² adj	0.7841			

It was possible to better observe the effects of the factors on the yield and volumetric productivity of propionic acid using response surface methodology. Figure 2b shows that that the highest yield was achieved when the substrate was only composed of corn steep liquor. Regarding productivity (Figure 2a), the same occurred in relation to animal feed effluent, i.e. productivity decreased.

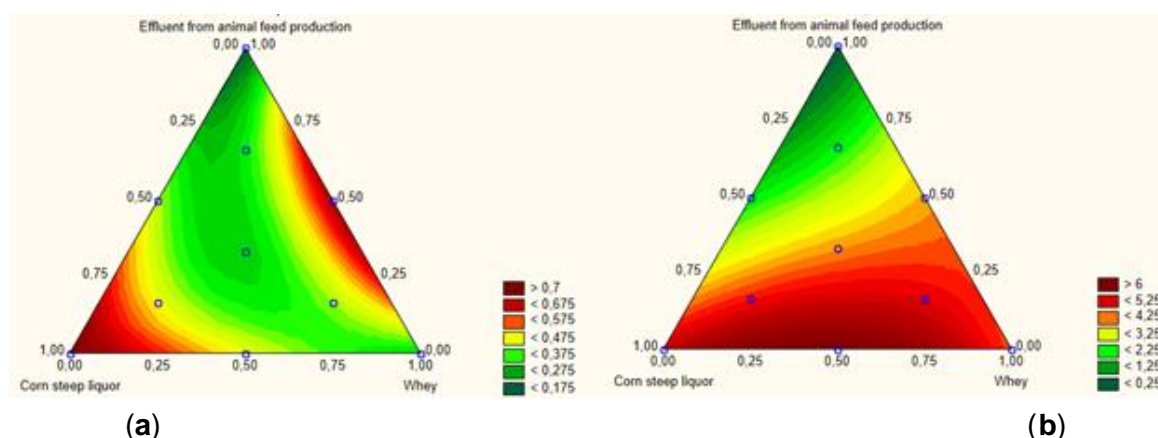


Figure 2. Response surfaces: **(a)** Response surfaces for volumetric productivity; **(b)** Response surfaces for yield.

DISCUSSION

In spite of the distinct characterisation results, the three effluents present in common COD and ammoniacal nitrogen levels were outside the release standards required by CONAMA Resolution No. 430/2011 and SEMA Resolution No. 021/2009, with COD limits of up to 225 mg. L⁻¹ and total ammoniacal nitrogen of 20 mg.L⁻¹. For pH (between 5 and 9), only the corn steep liquor was outside the limit established by Brazilian legislation [18,19]. Thus, the effluent needs to be treated before being discharged into rivers. The reuse of these effluents as a source of nutrients is promising because, in addition to valuing a by-product that was previously considered as waste, it is also possible to reduce the costs of treating effluent, either by reducing the volume of effluent to be treated, and/or the quantity of chemical reagents and number of steps for treating it.

In the first 24 hours of HRT, the pH values decreased in all the assays. The drop in pH indicated that organic acids were produced. Propionic bacteria have an optimum pH in the range of 6.0 to 7.0; below this pH range, cell growth and productivity may be adversely affected.

Nutrients must undergo transformations to be available for microorganisms. Lactose is a complex sugar (a disaccharide) and it is not directly metabolized, so the propionic bacteria, through the enzymes, hydrolyses the lactose, obtaining the monosaccharides galactose and glucose, which are substrates metabolisable by this bacterium. The glucose that is present is the most easily consumed sugar. Parizzi *et al.* [14] obtained the same results when using glucose for the production of propionic acid using different genetic strains of *Propionibacterium*. Table 3 shows that the lactic acid, if present, was completely consumed, demonstrating that it is a nutrient that was easily metabolised by the propionic culture. In the fermentative route, lactic acid is present in the later stage of glycolysis; thus, the bacteria seek shorter paths to obtain energy [20].

The presence of succinic acid is not desirable, because in the metabolic pathway of dicarboxylic acids, succinic acid may be a by-product, or succinyl-CoA-transferase enzyme can act with succinic acid, forming propionic acid. Therefore, if there is no activity on the part of this enzyme the yield of propionic acid may decrease. Even when the production of other organic acids occurred, the highest values were in relation to propionic acid, indicating that the conditions employed, as well as the nutrients present in the substrates, favoured the production of propionic acid by the propionic culture. This fact can be explained by the availability of necessary cofactors for the enzymes that catalyse the last steps in the metabolic pathway [21,22].

Table 5 shows that the highest yields of propionic acid were obtained in trials 4, 7 and 8. The substrates of these tests contained whey and corn steep liquor, with the proportions described in Table 2. Barbosa, Florentino, Florencio & Araújo [23] found that corn steep liquor and whey contained the micronutrients necessary for the development of microorganisms that can influence the production of propionic acid. Conversely, in the present paper, the tests that resulted in the lowest production of propionic acid were those that contained the highest concentration of animal feed effluent. The data presented in Table 5 demonstrates that propionic acid was produced in all the experiments. The yield from fermentation depends on several cultivate parameters, such as temperature, pH and the composition of the fermentative medium, which can be controlled. The productivity of propionic acid was low in all the tests. Jin and Yang [24] consider productivity values to be low when they are less than 1 g.L⁻¹.h⁻¹. Liu, Ma and Xu [25] obtained productivity of 0.23 g.L⁻¹.h⁻¹ using xylose as a carbon source with temperature at 30°C, initial pH of 6.0, and *P. acidipropionici* ATCC 4875 culture. Zhang *et al.* [26] used *P. acidipropionici* culture at a temperature of 32°C and automated pH control at 7.0, with the addition of 6N NaOH. A productivity of 0.21 g.L⁻¹.h⁻¹ was obtained, using glucose as a source of carbon and 0.07g.L⁻¹.h⁻¹ using glycerol; these values were considered to be low but they were higher than those found in the present study.

Pearson's correlation analysis showed a significant linear correlation between lactic acid consumption and propionic acid production ($r = 0.76$, $p = 0.010$), indicating that the

presence of lactic acid favoured the production of propionic acid. The presence of glucose, galactose and lactose, was not significant by correlation analysis and did not obtain high correlation values. Another interesting correlation was observed in relation to the production of acetic acid and the production of propionic acid ($r = 0.92$, $p < 0.001$), showing that the production of propionic acid was strongly correlated with the production of acetic acid. This reinforces the ratio that exists in the stoichiometric equation for the production of propionic acid because the molar ratio of propionic acid/acetic acid (P/A) in Test 8 for example was 2.57:1, which is close to the 2:1 molar ratio reported in the literature. Furthermore, the production of acetic acid generates ATP, which provides energy for the cellular metabolism of the propionic culture. The relationship between propionic acid production and yield was strong and significant ($r = 0.99$, $p < 0.001$), but the relationship with yield was low and non-significant ($r = 0.57$, $p = 0.087$).

The yield was better when corn steep liquor was present in the fermentation medium; however, when animal feed effluent was present the yield decreased. However, when either corn steep liquor or whey were present (or a mixture of them) there's an increase in productivity with significant positive effects. Because corn steep liquor showed significant positive effects for both yield and productivity, this by-product was chosen as the substrate for the next stage of planning.

The present paper showed that it was possible to produce propionic acid using *Propionibacterium acidipropionici* CCT 4843. The corn steep liquor was the best substrate that was evaluated and it presented the highest yield of 0.79 g.L^{-1} . The response surface plots showed that the use of whey and corn steep liquor was feasible to produce of propionic acid; however, only corn steep liquor improved the yield of propionic acid. The use of effluent from animal feed production was not favourable for the production of propionic acid.

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