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Isolation and characterization of bacterial producers of optically pure D(-) and L(+) lactic acid

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The aim of the present study was to isolate and characterize bacterial producers of optically pure D(-) and L(+) lactic acid for use in the synthesis of polymers employed in the production of resistive and biodegradable materials. Four hundred microorganisms were isolated. Of these strains, 20 had high capacity for production of only one lactic acid isomer which was selected for characterization. D(-) lactic acid producers were identified as *Weissella paramesenteroides* (25%), *Leuconostoc mesenteroides* (6.25%), *Leuconostoc lactis* (6.25%) and *Lactobacillus delbrueckii* (6.25%). L(+) lactic acid producers were identified as *Lactobacillus casei* (12.5%), *Enterococcus* sp. (18.75%), *Lactobacillus paracasei* (6.25%), *Streptococcus* sp. (6.25%) and *Lactobacillus* sp. (12.5%). The most promising isolate for L(+) lactic acid production was Ke8, in the presence of sucrose. Otherwise, Ke6 and Ke11 were the best producers, by using glucose as the carbon source. The best microorganism for the production of D(-) lactic acid was isolated from fermented milk (*L. delbrueckii* Y15C). However, the growth of this microorganism is easily inhibited on media containing sugar concentration greater than 60 g/L.

Key words: Lactic acid bacteria, identification, characterization, carbon sources, lactic acid, optimization.

INTRODUCTION

Lactic acid is used in the food industry as a preservative as well as for flavoring and acidulation, in the pharmaceutical industry for the production of cosmetics and ointments and in the chemical industry for the production of chemical bases (Wee et al., 2004). One of the most important applications of lactic acid is in the production of biodegradable materials, such as polylactate (PLA), which is used in the production of bags, computers components (Ohara, 2003), food packaging and plastic utensils. As PLA is bioabsorbable material, it is also employed in medicine in the regeneration of tissues, sutures, repairs and implants (Sakata et al., 2004). Polymers produced with D(-) and L(+) lactic acid have properties similar to plastics produced from fossil reserves, but offer the advantage of a high degree of biodegradability.

Lactic acid is produced through chemical synthesis or by microorganisms in fermentation processes. Fermentation is more efficient and economic, as it does not require high temperatures, high pressure or metallic catalysts. Moreover, energy consumption and the cost of raw materials are lower. It is possible to use renewable raw materials and work with microorganisms that produce a single isomer (Silva and Mancilha, 1991; Pandey et al., 2001), thereby offering an advantage over chemical synthesis, which always produces a racemic mixture.

The enantiopurity of lactic acid is an important factor in PLA polymer. The polymerization of a racemic mixture of L(+) or D(-) lactic acid leads to the synthesis of poly-DL-

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lactic acid, which is amorphous. The crystallinity rate and other important properties are controlled (Sodegard and Stolt, 2002), by the use of different concentrations of each isomer. Due to the chiral properties of lactic acid, the polymerization of L(+) lactic acid results in poly-L-lactic acid. Mixture with poly-D-lactic acid increases the melting point by 50°C, resulting in a highly regular complex with high degrees of crystallinity and thermal stability. In other words, in PLA, the ratio of L and D lactic acid influences the degradability of the polymers (Kharras et al., 1993), therefore it is easier to manufacture PLA with specific properties, for example, degradability, if L and D lactic acid are supplied separately. This kind of polymer is more stable than the amorphous polymer obtained with a racemic mixture (Lipinsky and Sinclair, 1986; Hofvendahl and Hahn-Hagerdal, 1997).

Pure isomers, L or D lactic acid, are more valuable than the racemic DL form because each isomer has its own applications in the cosmetics and pharmaceuticals industries. Lactic acid bacteria that have industrial potential should be homofermentative, such as *Lactococcus* and *Streptococcus,* which produces two molecules of lactic acid from one molecule of glucose. Heterofermentative lactic acid bacteria, *Leuconostoc* and *Weissella*, transforms glucose into lactate, ethanol and carbonic dioxide (Jay, 2000; Kuipers et al., 2000; Caplice and Fitzgerald, 1999) and are therefore deemed to be lower-yielding strains. Recovery of lactic acid and subsequent purification would also be easier in a homofermentative process. Other desirable properties for an industrially-useful lactic acid bacteria are the synthesis of pure isomers and tolerance to high product concentration and high temperature (42°C or higher) as these external conditions themselves would be useful to minimize contamination of the culture by other microorganisms (Tsai et al., 1993).

Lactic acid bacteria have specific morphological, metabolic and physiological characteristics, such as being Gram-positive, aerobic and facultative anaerobic, and a lack of spores. Such bacteria are either rod-shaped or coccus and are negative for catalase, oxidase, benzidine and gelatinase (Carr et al*.,* 2002).

The isolation and screening of microorganisms from natural sources has always been the most powerful means for obtaining useful and genetically-stable strains for industrially-important products (Adnan and Tan, 2007).

Proving the potential of isolated lactic acid bacteria, Wee et al. (2005), got 153.9 g l^{-1} of lactic production when 200 g l^{-1} of glucose and 15 g l^{-1} of yeast extract was used as carbon and nitrogen sources, respectively by *Lactobacillus* sp RKY2. When residues were used, Coelho et al. (2011a) obtained production of lactic acid of 98.4 g l^{-1} from molasses (193.5 g l^{-1}) and corn step liquor (37.50 ml l^{-1}) by *Lactobacillus plantarum* LMISM6.

The aim of the present study was to isolate, characterize and select potential bacterial producers of optically pure lactic acid, from renewable sources, for use in the production of durable and biodegradable materials.

MATERIALS AND METHODS

Sampling

For the isolation of lactic acid bacteria, samples were collected from the sugar and alcohol industry (filter cake, bagasse, molasses, sugarcane juice and cassava water), dairy industry (whey, kefir, yogurt and cottage cheese) and food industry (bacon, salami, sausage and pork) as well as ox rumen and silage. The samples were stored in a sterile container and transferred to Industrial Microbiology Laboratory.

Isolation of microorganism

The samples were cultivated in Man, Rogosa and Sharpe broth. This medium (18 ml) was inoculated with 2 ml of sample and incubated at 35°C and 150 rpm for 24 h. Serial dilutions with saline solution were made with the fermented broth. The bacteria were isolated and purified through repeated isolation in Petri dish with MRS broth solid. Purity was checked by examination under a microscope, after Gram coloration.

The strains were stored in a Man, Rogosa and Sharpe broth medium with 40% (v/v) glycerol at -80°C.

Microorganism selection

The 400 isolated microorganisms were tested for their lactic acid fermentation characteristics.

Fermentation with different carbon sources

Fermentation was performed with the best lactic acid producers to study the effect of different carbon sources (glucose, lactose and sucrose) on lactic acid production. The medium had the following composition (g l^{-1}): carbon source (60), peptone (10), yeast extract (5), meat extract (10), K_2HPO_4 (5.0), $MgSO_4.7H_2O$ (0.1), $MnSO_4.4H_2O$ (0.05), $CaCO_3$ (30.0) and 1 ml l^{-1} of Tween 80. Fermentations were carried out at 35°C for 48 h without shaking, in flasks with 5 mL of medium with 10% of inoculum. The pH was adjusted to 7.0 before autoclaving.

Strain characterization

Characterizations were performed with the best (Yp/s > 50%) lactic acid-producing microorganisms, using Gram staining, the catalase test, the oxidase cytochrome test and growth test under different conditions (45 and 10°C; pH 9.6; 6.5% and 18% NaCl). Inverted Durham tubes were used for the detection of CO_2 production from glucose.

For 16S rDNA sequence identification, chromosomal DNA was extracted and the 16S rDNA was amplified by polymerase chain reaction (PCR) following the methods described by Martins et al. (2007). Gene amplification of 16S rDNA was performed through PCR. The Pure Taq Ready-To-Go kit (GE Healthcare) was used with a final volume of 25 µL and 5 pmol of each primer: 27F (5'-AGAGTTTGATCA/CTGGCTCAG-3') and 1492R (5'-TACGGT/CTACCTTGTTACGACTT-3') (Polz and Cavanaugh, 1998).

The thermocycler was adjusted for initial denaturation at 96°C for 2 min, followed by 28 cycles at 96°C for 45 s, 50°C for 30 s and 60°C for 4 min. The PCR products were sequenced in both directions using an automatic sequencer (ABI 3500, Applied Biosystems).

Table 1. Variables and levels used in Plackett-Burman design.

Variable	Code	Range and level	
		-1	+1
Sodium Acetate (g l^{-1})	X_1	0	10
$MgSO_4$ (g l^{-1})	X_2	0	0.4
$MnSO_4$ (g l^{-1})	X_3	0	0.2
Citrate (g l^{-1})	X_4	0	4
K_2HPO_4 (g $^{l-1}$)	X_5	0	4
Tween 80 (mL$l^{-1)}$	X_6	0	2
Cysteine (g l^{-1})	X_7	0	0.2

Table 2. Real values of the independent values coded.

Variable (g l^{-1})	Code	Range and level				
		-1.68	-1	0	1	1.68
Sugarcane juice	X_1	2.4	50	120	190	237.6
Corn Step liquor	X_2	3	20	45	70	87
K_2HPO_4	X_3	0.64	2.0	4.0	6.0	7.36

Forward and reverse sequences were aligned and edited with the BioEdit program (Hall, 1999). The isolates were identified at the species level and compared with deposit sequences in the GenBank using the BLAST (www.ncbi.nlm.nih.gov/blast).

Production of L (+) lactic acid by *Lactobacillus casei* Ke8 from different substrates

Microorganisms

The microorganism *L. casei* Ke8 were isolated from Keffir and they were excellent L(+) lactic acid producers. The strain was stored in MRS broth with 20% (v/v) glycerol at -20°C.

Plackett-Burman experimental design

The purpose of this first step of the optimization was to identify the medium components that have significant effect on lactic acid production by *L. casei* Ke8. The medium was composed of sugarcane juice and corn step liquor (36.75 ml l^{-1}) as nitrogen source. Twelve experiments were generated from 7 factors: sodium acetate, magnesium sulphate, manganese sulphate, ammonium citrate, potassium phosphate, Tween 80 and cysteine. The variables with confidence level greater than 95% were considered to have significant influence on lactic acid production. The PB experimental design was based on the first-order model with no interaction among the factors. The concentrations used for each variable are shown in Table 1.

A central composite design (CCD) was made with the variables that significantly increased production of lactic acid.

Central composite design and optimization by response surface methodology

In this process, the variable concentrations were optimized: sugarcane juice, nitrogen source and potassium phosphate for L(+) lactic acid production by the microorganism, *L. casei* Ke8. For this, de central composite design was made with four replicates at center points. The independent variables of the experiments were coded according to the following equation:

$$X_n = \frac{\left(X - X_0\right)}{\frac{X_{+1} - X_{-1}}{2}} \quad (1)$$

Where X_n is the coded value of variable in the experiment, X is the real value of a variable to be calculated, X_0 is the real value of a variable at the center point, X_{+1} is the real value of a variable in the superior level and X_{-1} is the real value of a variable in the inferior level.

The levels used for the codification of the independent variables are show in the Table 2. Samples of 1 ml were taken from the fermentation broth after 48 h of fermentation and were centrifuged and analyzed for lactic acid production.

Analytical methods

Lactic acid and sugar concentrations were determined using a high-performance liquid chromatography system (HPLC) equipped with a UV detector at 210 nm. A Rezex ROA (300 × 7.8 mm, phenomenex) column was eluted with 5 mM H_2SO_4 as a mobile phase at a flow rate of 0.6 mL/min and the column temperature was maintained at 60°C. Optical purity of D(−) and L(+) lactic acid was determined with HPLC using a chirex 3126 phenomenex (150 × 4.6 mm) column with 1 mM of $CuSO_4$ as the mobile phase at 1 mL/min (30°C).

For sugars the HPLC system were equipped with IR detector, a rezex RMN carboidrat Na+ Phenomenex (300X 7,8 mm) column was eluted with water ultrapure as mobile phase at flow rate of 0,6 mL min^{-1} and column temperature was 40ºC.

Statistical analyses

The software package Statistica 7.0 (Stat Soft, USA) was used for

Table 3. Yields specified per strain for each carbon source.

Isolated	Glucose	Lactose	Sucrose
	$Y_{(P/S)}$	$Y_{(P/S)}$	$Y_{(P/S)}$
Ke1	0.57	0.2593333	0.076333
Ke2	0.901833	0.8041667	0.836667
Ke3	0.61	0.2233333	0.0555
Ke5	0.477333	0.2785	0.129833
Ke6	0.670667	0.2793333	0.054333
Ke7	0.734	0.5368333	0.8805
Ke8	0.706	0.5605	0.974833
Ke9	0.602	0.4291667	0.076667
Ke 11	0.985833	0.77	0.959667
Ke12	0.495333	0.244	0.091833
Ke13	0.586	0.2435	0.068833
Ke15	0.437333	0.287	0.1035
Ke19	0.99	0.6663333	0.2425
Ke20	0.583333	0.1876667	0.093167
Ke23	0.581333	0.2231667	0.048
Ke24	0.555	0.84	0.314833
Ke25	0.650667	0.247	0.052333
Ke27	0.708333	0.2158333	0.080333
Ke32	0.597667	0.403	0.073833
Tort 4	0.754667	0.5186667	0.216333
Tort 10	0.65	0.0755	0.417
Baga 3	0.922333	0.8098333	0.933833
V9	0.583333	0.086	0.052
V35	0.463333	0.0726667	0.072667
Ch17	0.9465	0.607	0.195333
Y2	0.322667	0.3156667	0.216
Y16f	0.616	0.2733333	0.254
Y16B	0.471333	0.3256667	0.333667
Y4c	0.526167	0.2916667	0.383333
Y15C	0.672	0.4686667	0.664667
Y15A	0.665333	0.6516667	0.715
Y5N	0.645	0.377	0.5445
Y15i	0.637667	0.5606667	0.598
Y4B	0.294	0.3263333	0.094333
Y4A	0.207667	0	0.090667
CC28	0.545167	0.06	0.182167
CC29	0.629667	0.0428333	0.1955
CC10	0.577667	0.0595	0.181167
Bslm14	0.422667	0.0973333	0.229833
CH25	0.468	0.0488333	0.184833
CH24	0.412333	0.0763333	0.204

for experimental design and regression analysis of the experimental data. The response surface was generated to understand the interaction among the variables. The optimal points for the variables were obtained from Maple 9.5 (Waterloo Maple Inc., Ontario, Canada). The behavior of the system was explained by the following quadratic equation:

$$Y = b_0 + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum b_{ij} x_i x_j \quad (2)$$

Where Y is the predicted response, that is, lactic acid concentration; b_0 is the offset term, b_i is the linear effect; b_{ii} is the squared effect; b_{ij} is the interaction effect and x_i is the independent variable.

To Equation (2) was applied the results and were made a statistical evaluation to estimate the parameters between the values for T of Student for each parameters and were eliminated with the results with significance level (p) superior to 5%, that is, the variables related to this results were considered not relevant when the p value is superior to 5%. The non significant parameters were eliminated, and one equation which represents the effects of the variables in the production of lactic acid were obtained. It is also possible to predict the better condition for the process of lactic acid production. The R^2 value and the comparison between the F calculated and the F tabulated were used for finding the significance or non significance of the model.

RESULTS AND DISCUSSION

Isolation and selection of microorganisms

Among the 400 microorganisms isolated from different samples, 34 were from cassava, 101 were from sugarcane processing (filter cake, bagasse, sugarcane juice and molasses), 13 were from sauerkraut, 22 were from ox rumen, 19 were from silage, 77 were from dairy products (catupiry cheese, kefir, whey and yogurt), 99 were from meat (bacon, pork and sausage) and 35 were from wine. No lactic acid bacteria were found in corn step liquor. This may be due to the high concentration of nitrogen in the sample, which may have inhibited the growth of microorganisms. According to De Lima et al. (2009), high nitrogen concentrations may cause cell death in bacteria.

Only 19 of the 400 microorganisms did not produce lactic acid. One hundred and twenty-one produced lactic acid with yield product/substrate (Yp/s) equal to or greater than 0.5 (Table 3). The microorganisms with the highest production of lactic acid (Yp/s ≥ 0.85) were isolated from cassava (Lmism1, Lmism6, AMP1, AMP3 and AMP6), sugarcane bagasse (BSLA9 and Baga3), filter cake (Tort 4), sausage (SA 10) and kefir (Ke8, Ke11 and Ke19).

The 121 isolates were analyzed with regards to the production of optically pure lactic acid: 80 produced a racemic mixture, 29 produced L(+) lactic acid and 12 produced D(-) lactic acid. These findings are in agreement with data reported by Garvie (1967), Manome et al. (1998) and Costa et al. (2008), who also found a greater number of producers of racemic lactic acid in the environment. L-lactate dehydrogenase and D- lactate dehydrogenase enzymes determine the chirality of the lactate formed (Garvie, 1980).

The enzymatic reduction of pyruvate to lactate is processed by stereospecific dehydrogenases. Bacteria are classified as either L(+) or D(-) when the production of L(+) or D(-) lactic acid is greater than 75%, respectively. Bacteria with the equal production of both isomers are designated DL bacteria (Manome et al., 1998). The proportion between the isomers during fermentation varies with the genus and species of microorganism. L(+) lactic acid is produced by *Aerococcus, Carnobacterium, Enterococcus, Lactococcus, Tetragenococcus, Streptococ-*

cus and *Vagococcus* and D(-) lactic acid is produced by *Leuconostoc*, *Oenococcus* and *Lactobacillus, Pediococcus* and *Weissella* which produce both isomeric forms either separately or together, depending on the species and culture conditions (Liu, 2003).

L(+) lactic acid bacteria predominated in the kefir samples. Cho and Ki Do (2006) reported a similar finding in samples of Jeotgal (a traditional Korean food).

The 41 isolates with optical purity (29 L(+) lactic acid producers and 12 D(-) lactic acid producers) were submitted to different carbon sources fermentation (glucose, sucrose and lactose) to determine the best carbon source for metabolism (Table 4). The isolates exhibited enhanced lactic acid production when glucose was the carbon source. Wee et al. (2005) evaluated lactic acid production by *Lactobacillus* sp. RKY2 using different carbon sources (glucose, fructose, maltose, galactose, lactose, glycerol, xylose, sucrose and starch) and found that the highest yield was obtained with glucose, followed by fructose and maltose. When the authors used 30 g l^{-1} of glycerol, starch and glucose lactic acid production was 2.24, 1.19 and 9.7 g l^{-1}, respectively. Table 4 demonstrates that the choice of microorganism for lactic acid production depends mainly on the substrate used in the fermentation process.

The most promising isolates for L(+) lactic acid production were Ke6 and Ke11 using glucose as the carbon source. The isolate Ke8 produced well in the presence of sucrose. Regarding the production of D(-) lactic acid, the isolate Y15C achieved the best production with sucrose, glucose or lactose as the carbon source. According to Kascak et al. (1996), *Lactobacillus delbrueckii* prefers glucose or lactose as the substrate, while *Lactobacillus bulgaricus* prefers lactose.

The proportion of lactic acid isomers varied according to the carbon source (Table 4). According to Hofvendahl and Hahn-Hagerdal (2000), the amount of the predominant isomer can be increased with an increase in pH and the amount of substrate. However, high temperatures and uncontrolled pH are unfavorable to the production of any isomer.

Bai et al. (2003) worked on *Lactobacillus lactis* growing on three different carbon sources: glucose, xylose and lactose at concentration of 50 g l^{-1}. The highest L(+) lactic acid production obtained a yield of 100% with glucose, followed by xylose, 72% and lactose, 48%.

The microorganisms selected in the present study demonstrate considerable potential for use in a variety of industries especially those isolated from kefir and yogurt, due to the high concentration of optically pure lactic acid produced.

Ding and Tan (2006), using several feeding strategies, in bioreactor, for L(+) lactic acid production by *L. casei* using glucose as a carbon source observed that the best strategy was the exponential fermentation. The maxim production of lactic acid was 180 g l^{-1} with glucose (850 g l^{-1}) and yeast extract (1%) as feeding source.

Strain characterization

The 16 most promising strains based on greater lactic acid production and high degree of optical purity, were identified using biochemical and molecular techniques. Seven were D(-) lactic acid producers and nine were L(+) lactic acid producers (Tables 5 and 6).

D(-) lactic acid bacteria were identified as *Weissella paramesenteroides* (25%), *Leuconostoc mesenteroides* (6.25%), *Leuconostoc lactis* (6.25%) and *L. delbrueckii* (6.25%). L(+) lactic acid bacteria were identified as *L. casei* (12.5%), *Enterococcus* sp. (18,75%), *Lactobacillus paracasei* (6.25%), *Streptococcus* sp. (6.25%) and *Lactobacillus* sp. (12.5%).

L. casei, *L. paracasei* subsp *paracasei* and *Enterococcus* sp. were isolated from kefir in this work. Lactic acid bacteria were the most frequent microorganisms in Brazilian kefir and the most frequent species were *L. paracasei*, *Lactobacillus parabuchnei* and *L. casei*. No isolates belonging to the genus *Enterococcus* were found according to Magalhães et al. (2011). *Lactobacillus* sp, *Weissella mesenteroides* and *L. mesenteroides* were isolated from sauerkraut. *L. mesenteroides* and *W. paramesenteroides* were isolated from sugarcane juice and sugarcane bagasse. *L. delbrueckii* ssp. *delbrueckii* were isolated from fermented milk. These findings are similar to those reported by Mundt et al. (1967), who found that *Leuconostoc* was the predominant genus in vegetables, such peas, beans, cucumber, okra and cachaça (alcohol made from sugarcane in Brazil), Gomes et al. (2010) found *L. plantarum* and *L. casei* to be the predominant species. Isolating lactic acid bacteria from yogurt, Ali (2011) found predominantly the genus *Leuconostoc,* followed by *Lactococcus*.

The production of optically pure acid lactic isomers is considerable important to production of PLA. Polymer characteristics, such as crystallinity, are influenced by the optical composition of lactic acid.

Polymers formed from a mix of both isomers are more resistant to temperature, with an approximate 50°C increase in the fusion point when compared with polymers made from only one isomer.

The incorporation of different proportions of lactic acid isomers allows different applications for polymers, such as in the composition of drug-carrier microspheres and implants as well as replacement materials for composites made from petroleum in resins, packaging and fibers (Lunt, 1998; Henton et al., 2005). Some of the isolates analyzed in the present study demonstrate considerable potential for use in PLA production, as the strains isolated produce optically pure lactic acid isomers. Moreover, this product can be produced by bacteria from renewable sources.

Plackett-Burman experimental design

The design matrix of Plackett-Burman design (real and

Table 4. Production of lactic acid and proportion of enantiomers produced by microorganisms (initial concentration: 60 g l^{-1}).

Isolated	Glucose			Lactose			Sucrose		
	Total LA[a]	L-(+)[b]	D-(-)	Total LA	L-(+)	D-(-)	Total LA	L-(+)	D-(-)
Ke1	34.20	100.00	0.00	15.56	100.00	0.00	4.58	87.12	12.88
Ke2	54.11	97.17	2.83	48.25	94.32	5.68	50.20	100.00	0.00
Ke3	36.60	100.00	0.00	13.40	100.00	0.00	3.33	100.00	0.00
Ke5	28.64	100.00	0.00	16.71	100.00	0.00	7.79	85.49	14.51
Ke6	40.24	100.00	0.00	16.76	100.00	0.00	3.26	100.00	0.00
Ke7	44.04	90.01	9.99	32.21	95.34	4.66	52.83	95.80	4.20
Ke8	42.36	95.80	4.20	33.63	95.51	4.49	58.49	100.00	0.00
Ke9	36.12	97.23	2.77	25.75	100.00	0.00	4.60	82.61	17.39
Ke 11	59.15	98.00	2.00	46.20	93.57	6.43	57.58	93.82	6.18
Ke12	29.72	100.00	0.00	14.64	100.00	0.00	5.51	80.04	19.96
Ke13	35.16	95.22	4.78	14.61	100.00	0.00	4.13	79.66	20.34
Ke15	26.24	100.00	0.00	17.22	100.00	0.00	6.21	77.29	22.71
Ke19	59.40	92.56	7.44	39.98	93.42	6.58	14.55	86.25	13.75
Ke20	35.00	97.54	2.46	11.26	100.00	0.00	5.59	74.96	25.04
Ke23	34.88	95.81	4.19	13.39	100.00	0.00	2.88	100.00	0.00
Ke24	33.30	100.00	0.00	50.40	94.31	5.69	18.89	88.46	11.54
Ke25	39.04	97.44	2.56	14.82	100.00	0.00	3.14	100.00	0.00
Ke27	42.50	97.60	2.40	12.95	100.00	0.00	4.82	90.04	9.96
Ke32	35.86	100.00	0.00	24.18	92.64	7.36	4.43	82.17	17.83
Tort 4	45.28	95.32	4.68	31.12	95.85	4.15	12.98	90.83	9.17
Tort 10	39.00	100.00	0.00	4.53	81.90	18.10	25.02	100.00	0.00
Baga 3	55.34	94.49	5.51	48.59	94.38	5.62	56.03	94.49	5.51
V9	35.00	91.17	8.83	5.16	84.30	15.70	3.12	72.76	27.24
V35	27.80	92.88	7.12	4.36	78.44	21.56	4.36	88.30	11.70
Ch17	56.79	91.20	8.80	36.42	95.14	4.86	11.72	92.49	7.51
Y2	19.36	100.00	0.00	18.94	94.03	5.97	12.96	100.00	0.00
Y16f	36.96	100.00	0.00	16.40	100.00	0.00	15.24	100.00	0.00
Y16B	28.28	91.51	8.49	19.54	100.00	0.00	20.02	95.70	4.30
Y4c	31.57	89.83	10.17	17.50	95.23	4.77	23.00	94.26	5.74
Y15C	40.32	2.78	97.22	28.12	0.00	100.00	39.88	0.00	100.00
Y15A	39.92	5.01	94.99	39.10	4.60	95.40	42.90	3.68	96.32
Y5N	38.70	3.51	96.49	22.62	2.83	97.17	32.67	2.57	97.43
Y15i	38.26	0.00	100.00	33.64	4.34	95.66	35.88	8.97	91.03
Y4B	17.64	9.86	90.14	19.58	7.97	92.03	5.66	8.13	91.87
Y4A	12.46	10.59	89.41	0.00	0.00	0.00	5.44	6.99	93.01
CC28	32.71	1.65	98.35	3.60	11.39	88.61	10.93	3.11	96.89
CC29	37.78	1.22	98.78	2.57	10.51	89.49	11.73	11.85	88.15
CC10	34.66	6.87	93.13	3.57	10.92	89.08	10.87	5.06	94.94
Bslm14	25.36	1.18	98.82	5.84	15.41	84.59	13.79	4.13	95.87
CH25	28.08	3.35	96.65	2.93	10.58	89.42	11.09	3.16	96.84
CH24	24.74	2.43	97.57	4.58	20.31	79.69	12.24	3.02	96.98

[a] Total LA: Total lactic acid; [b] Proportion of lactic acid produced in %.

coded values) of 15 experiments from seven variables added to sugar cane juice (X_1 = acetate, X_2 = $MgSO_4$, X_3 = $MnSO_4$, X_4 = citrate, X_5= K_2HPO_4, X_6 = Tween 80, X_7 = cysteine), with the respective result (lactic acid), is presented in Table 7.

K_2HPO_4 had a significant positive effect on lactic acid production, on the confidence level of 95%. This fact can be better seen in Figure 1 (Pareto chart), where the effects of the variables are represented. According to Honorato et al. (2007), the addition of phosphate in the culture me-

Table 5. Biochemical and physiological characteristics of selected strains.

Isolate[a]	45°C	10°C	6.5% NaCl	18 % NaCl	pH 9.6	CO_2	Isomer	Morphology	Genus
Ke6	+[b]	+	+	-	+	-	L-(+)	Coccus	*Enterococcus*
Ke8	+	+	+	-	-	-	L-(+)	Rod	*Lactobacillus*
Ke11	-	+	-	-	-	-	L-(+)	Rod	*Lactobacillus*
Ke27	+	+	+	-	+	-	L-(+)	Coccus	*Enterococcus*
Ke1	+	+	+	-	+	-	L-(+)	Coccus	*Enterococcus*
Tort10	+	-	-	-	-	-	L-(+)	Coccus	*Streptococcus*
Ke7	+	+	+	-	-	-	L-(+)	Rod	*Lactobacillus*
CH17	+	-	-	-	-	-	L-(+)	Rod	*Lactobacillus*
Ke2	+	+	+	-	-	-	L-(+)	Rod	*Lactobacillus*
CH25	-	-	+	-	-	+	D-(-)	Coccus	*Leuconostoc/Weissella*
CC28	-	+	+	-	-	+	D-(-)	Coccus	*Leuconostoc/Weissella*
Bslm14	-	+	+	-	-	+	D-(-)	Coccus	*Leuconostoc/Weissella*
CH24	-	+	+	-	-	+	D-(-)	Coccus	*Leuconostoc/Weissella*
CC29	-	+	+	-	-	+	D-(-)	Coccus	*Leuconostoc/Weissella*
CC10	-	+	+	-	-	+	D-(-)	Coccus	*Leuconostoc/Weissella*
Y15C	+	-	+	-	-	-	D-(-)	Rod	*Lactobacillus*

[a] None of the isolates presented motility, endospores, catalase or oxidase cytochrome; all were Gram +; [b]+: characteristic present; -: characteristic absent

Table 6. Identification of isolates according to sequence obtained in comparisons with GenBank data.

Isolate	Accession No. in GenBank of corresponding sequence	Homology with sequence in GenBank (%)	Identification
Ke11	NR 07032.1	97	*Lactobacillus casei*
Ke6	HM462408.1	97	*Enterococcus* sp.
Ke27	FJ917736.1	96	*Enterococcus* sp.
Ke1	HM058837.1	96	*Enterococcus* sp.
Ke7	AY773951.1	97	*Lactobacillus paracasei*
Ke8	NR 075032.1	99	*Lactobacillus casei*
CH17	HM162419.1	99	*Lactobacillus* sp.
Tort10	AB489099.1	99	*Streptococcus* sp.
Ke2	HM162415.1	100	*Lactobacillus casei*
CH25	GQ466165.1	98	*Leuconostoc mesenteroides*
CC28	FJ405229.1	97	*Weissella paramesenteroides*
Bslm14	GU344717.1	98	*Leuconostoc lactis*
CH24	FJ538548.1	99	*Weissella paramesenteroides*
CC29	FJ538548.1	99	*Weissella paramesenteroides*
CC10	FJ405229.1	99	*Weissella paramesenteroides*
Y15C	AB680027.1	98	*Lactobacillus delbrueckii* subsp. *delbrueckii*

medium increases the growth of the microorganism and the lactic production, since this component keeps the pH constant, allowing the conduction of fermentation for a longer time, and the pH near the optimal value of growth.

For both microorganisms, the K_2HPO_4 exercised a positive effect, with a confidence level significant of 95%. This means that when concentration or the range of values increases for the level -1 to +1, increases in the lactic acid concentration occurs. So, these variables were used together with carbon and nitrogen source variables for the optimization of lactic acid production by using central composite design.

Optimization of the production of L(+) lactic acid by central composite design

The influence of the variables, sugarcane juice (X_1), nitrogen source (X_2) and K_2HPO_4 (X_3) in production of L(+) lactic acid by *L. casei* Ke8 were studied following a factorial central composite design of four replicates at the

Table 7. Plackett-Burman design (real and coded values) with the respective result for the production of lactic acid.

Run	Independent variable							Result
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Lactic acid (g l^{-1}) Ke8
1	10 (1)*	0 (-1)*	0.2 (1)	0 (-1)	0 (-1)	0 (-1)	0.6 (1)	25.9
2	10 (1)	0.4 (1)	0 (-1)	4 (1)	0 (-1)	0 (-1)	0 (-1)	8
3	0 (-1)	0.4 (1)	0.2 (1)	0 (-1)	4 (1)	0 (-1)	0 (-1)	50.85
4	10 (1)	0 (-1)	0.2 (1)	4 (1)	0 (-1)	2 (1)	0 (-1)	9.35
5	10 (1)	0.4 (1)	0 (-1)	4 (1)	4 (1)	0 (-1)	0.6 (1)	42.2
6	10 (1)	0.4 (1)	0.2 (1)	0 (-1)	4 (1)	2 (1)	0 (-1)	56.4
7	0 (-1)	0.4 (1)	0.2 (1)	4 (1)	0 (-1)	2 (1)	0.6 (1)	13.9
8	0 (-1)	0 (-1)	0.2 (1)	4 (1)	4 (1)	0 (-1)	0.6 (1)	47.7
9	0 (-1)	0 (-1)	0 (-1)	4 (1)	4 (1)	2 (1)	0 (-1)	61.1
10	10 (1)	0 (-1)	0 (-1)	0 (-1)	4 (1)	2 (1)	0.6 (1)	57.7
11	0 (-1)	0.4 (1)	0 (-1)	0 (-1)	0 (-1)	2 (1)	0.6 (1)	65.25
12	0 (-1)	0 (-1)	0 (-1)	0 (-1)	0 (-1)	0 (-1)	0 (-1)	43.15
13	5 (0)	0.2 (0)	0.1 (0)	2 (0)	2 (0)	1 (0)	0.3 (0)	69.5
14	5 (0)	0.2 (0)	0.1 (0)	2 (0)	2 (0)	1(0)	0.3 (0)	65.5
15	5 (0)	0.2(0)	0.1 (0)	2 (0)	2 (0)	1 (0)	0.3 (0)	70.3

X_1 = Acetate, X_2 = $MgSO_4$, X_3 = $MnSO_4$, X_4 = citrate, X_5 = K_2HPO_4 X_6 = Tween 80, X_7 =cysteine; *(-1) and (1) are coded levels.

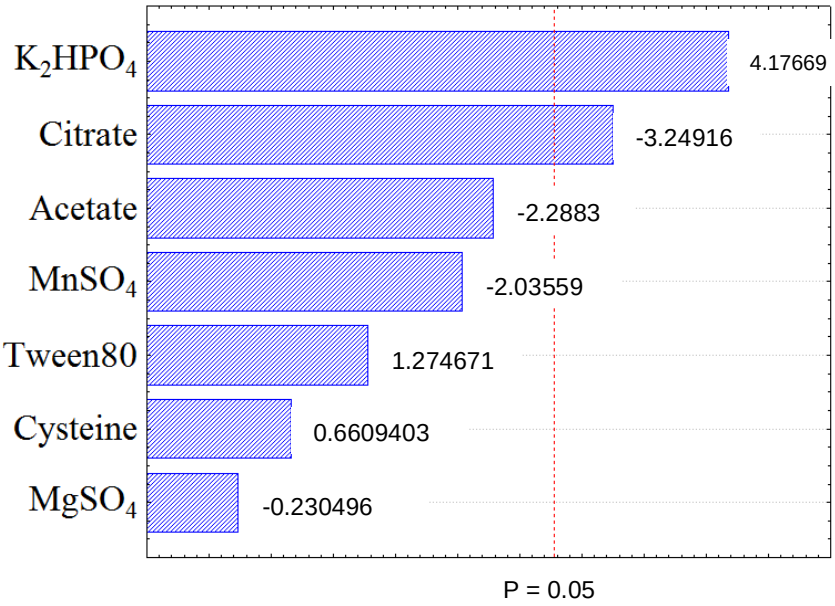

Figure 1. Pareto chart for lactic acid production by *L. casei* Ke8.

center points. Table 8 shows the lactic acid production and the factors used in the design.

The higher production of L(+) lactic acid was 70.13 g l^{-1} obtained from 237.6 g l^{-1} of reducing sugars from the sugarcane juice, 45 g l^{-1} of CSL and 4 g l^{-1} of K_2HPO_4. From the experimental data, shown in Table 8, we applied multiple regression analysis methods: Production of lactic acid by *L. casei* Ke 8:

$$Y=57,57+18,22X_1+14,15X_2-5,07X_3-1,622X_1X_2-4,99X_1X_3+3,10X_2X_3-6,99X_1X_1-7,13X_2X_2-5,39X_3X_3 \quad (3)$$

Where Y is the predicted response, that is, the lactic acid concentration, and X_1, X_2 and X_3 are the coded values of the test variables sugar cane juice and nitrogen source and K_2HPO_4 respectively.

The response surface quadratic model was performed in

Table 8. Coded and real (g l^{-1}) values of central composite design and lactic acid production.

Run	Sugarcane Juice	Nitrogen souce	K_2HPO_4	Lactic acid
1	-1(50)	-1(20)	-1(2)	15.16
2	-1(50)	-1(20)	1(6)	5.9
3	-1(50)	1(70)	-1(2)	36.6
4	-1(50)	1(70)	1(6)	32.7
5	1(190)	-1(20)	-1(2)	65.4
6	1(190)	-1(20)	1(6)	29.1
7	1(190)	1(70)	-1(2)	73.3
8	1(190)	1(70)	1(6)	56.46
9	-1.68(2.4)	0(45)	0(4)	1.8
10	1.68(237.6)	0(45)	0(4)	70.13
11	0(120)	-1.68(3)	0(4)	2.96
12	0(120)	1.68(87)	0(4)	68.23
13	0(120)	0(45)	1.68(0.64)	41.4
14	0(120)	0(45)	1.68(7.36)	39.6
15	0(120)	0(45)	0(4)	56.2
16	0(120)	0(45)	0(4)	55.86
17	0(120)	0(45)	0(4)	65.43
18	0(120)	0(45)	0(4)	53.4

Table 9. Analysis of variance for the quadratic model.

Source	Sum of squares	Degree of freedom	Mean square	F_{-value}	P > F
Model	9080.535	9	1008.948	12.42935	0.000824
Error	649.397	8	81.175		
Lack of fit	565.521	5	113.104	4.0454	0.139715
Pure error	83.876	3	27.959		
Total	9729.932	17			

R^2 = 0.93; Adjusted R^2 = 0.86; R = 0.96.

in the form of analysis of variance (ANOVA) and the results are summarized in Table 9. The Fisher's F-test was used to check the statistical significance of Equation 3. The ANOVA of the quadratic regression model for the data of *L. casei* Ke8 production also demonstrates that the model is highly significant, as is evident from the Fisher test (F_{calc} (9,8) = 12,42935 > Ft(9,8) = 3,388 and has a very low probability value (p < 0.000824). The fit of the model was checked by determination coefficient (R^2) and the multiple correlation coefficient R. In this case, the value of the R^2 (0.93) for Equation 3 indicates that the sample variation of 93% was explained by the model equation. The value of the adjusted determination coefficient (adjusted R^2 = 0.86) is also high, which indicates high significance of the model.

The results are consistent with the assumptions of the statistical model, in other words, the errors exhibit independence and there are normalities in the results.

The student's t-distribution and the probability (P) values served as a tool to check the significance of each of the coefficients, which in turn may represent the pattern of the interaction between the test variables. The smaller the magnitude of the P value, the more significant the corresponding coefficient.

It is evident from Table 10 that the independent variables (X_1, X_2 and X_1^2) had significant effect (observed through *P*-valor), and the variables X_1, X_2 had a positive effect, then, an increase in the concentration led to an increase response (lactic acid).

The data of production by *L. casei* Ke8 showed that the independent variables X_1 (sugar cane juice) and X_2 (CSL)) had significant effect (observed through *P*-valor), increasing the production until it reached a certain concentration, because the quadrate variables $X_1^2$and X_2^2 had a negative effect, it means, high concentration of this variables could reduce the production. The 3D response surface is the graphical representation of the regression Equation 3 plotted to understand the interaction of the variables and to locate the optimum level of each variable for maximum response. From Equation 3, the response

Table 10. The least-squares fit and parameter estimates.

Term	Estimate	Standard error	T	Pr>|t|
Intercept	57.56807	4.498288	12.79777	0.000001
X_1	18.21837	2.437882	7.47303	0.000071
X_2	14.15146	2.437882	5.80482	0.000403
X_3	-5.07588	2.437882	-2.08208	0.070885
X_1X_2	-1.62250	3.185409	-0.50935	0.624254
X_1X_3	-4.99750	3.185409	-1.56887	0.155316
X_2X_3	3.10250	3.185409	0.97397	0.358596
X_1^2	-6.99706	2.532825	-2.76255	0.024578
X_2^2	-7.12784	2.532825	-2.81419	0.022696
X_3^2	-5.39409	2.532825	-2.12967	0.065826

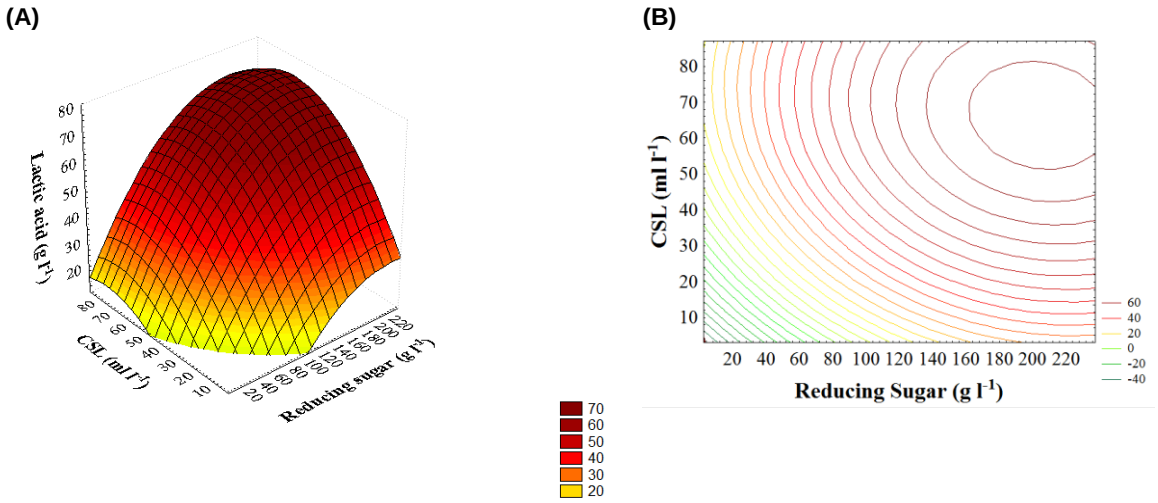

Figure 2. Response surface (A) and curve contour (B) for lactic acid production by *L. casei* Ke8 in function of the variables: sugar cane juice concentration and CSL.

surface for production of lactic acid was built, by *L. casei* Ke8, in function of the variables, total reducing sugar concentration of sugarcane juice (X_1) and corn step liquor (X_2) Figure 2.

From analyses of Figure 2, it is possible to deduce the high influence of the sugar cane juice, as well as the corn step liquor in the production of lactic acid. The great region for lactic acid production ranged between 160 and 240 g l^{-1} of total sugar reducers of sugar cane juice and between 50 and 80 g l^{-1} of CSL. In lower or higher concentrations of these values, decrease of lactic acid production occurs. Many studies shows that cell growing and lactic acid production by bacteria from the genera *Lactobacillus* are inhibited by substrate (Gonçalves et al., 1991; Burgos-rubio et al., 2000). Sule et al. (2004) reported that the maximum production of lactic acid was 21 g l^{-1}, using 50 g l^{-1} of sucrose, and the 100 g l^{-1} of sucrose resulted in decrease of lactic acid production.

Residues were used by Wee et al. (2006) for the production of L(+) lactic acid. The microorganism utilized was *Enterococcus faecalis* RKY1 and the carbon and nitrogen source was wood hydrolyzate (equivalent to 50 g l^{-1} of glucose) and corn step liquor, respectively. The production of lactic acid obtained was 48.6 g l^{-1}.

For D(-) lactic acid production, Coelho et al. (2011b) using sugar cane juice (116 g l^{-1}) as carbon source and yeast autolysed (44.25 g l^{-1}) as nitrogen source, reached a production of 66.11 g l^{-1} of D(-) lactic acid by *L. mesenteroides* B512.

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