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High D(−) lactic acid levels production by *Sporolactobacillus nakayamae* and an efficient purification

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Abstract This study is the first to report the efficient production of D(−) lactic acid by a strain of *Sporolactobacillus nakayamae* utilizing inexpensive peanut flour as a nitrogen source and commercial sucrose as a carbon source. A D(−) lactic acid concentration of 112.93 g/L was obtained by fermentation using 110.00 g/L of commercial sucrose, 150.40 g/L of peanut flour, and 0.16 ml/L of Tween 80. The optimal medium composition was determined using experimental design such as a Plackett-Burman, fractional factorial designs, and response surface methodology. The purification and recovery of D(−) lactic acid from the fermentation medium were performed using two purification techniques (filtration with activated carbon, followed by celite and cation resin exchange). This approach showed a high efficiency in protein and sugar removal as well in D(−) lactic acid recovery from the fermentation medium. Thus, the results indicate that *S. nakayamae* is a promising new D(−) lactic acid producer.

Introduction

The production of lactic acid by microorganisms has gained a prime position in industry because it is cost effective and eco-friendly. Lactic acid is a versatile chemical with a wide variety of applications across a range of industries (John et al. 2009; Djukić-Vuković et al. 2012). Lactic acid can be produced by fermentation from different raw materials and through various technological means; additionally, it has versatile properties as a precursor for various chemicals and materials (Kim et al. 2006; Zhang et al. 2007; Hetényi et al. 2008; Dumbrepatil et al. 2008).

Approximately 40 % of produced lactic acid is used in manufacturing polylactic acid (Bozell and Petersen 2010), which has recently been studied with great interest as a biodegradable polymer that can be used in the production of food packaging, plastic utensils, and plastic sheeting used for parts of computers, thereby replacing products made from petroleum (Ohara 2003; Coelho et al. 2011a, b). L(+) Lactic acid is used in the food industry to preserve human foodstuffs and is also utilized in the pharmaceutical and cosmetic industries (Li et al. 2004; Naveena et al. 2005; Wee et al. 2006a, b).

Some microorganisms can produce optically pure L(+) or D(−) lactic acid isomers via fermentation, while racemic DL-lactic acid is produced via chemical synthesis (Hofvendahl and Hahn-Hägerdal 2000; Lima et al. 2010). In the literature, there are more studies involving L (+) lactic acid than there are for D(−) lactic acid production, and additionally, the production yield of D(−) lactic acid is lower than that of L(+) lactic acid (Wang et al. 2011).

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Design experiments have been frequently used for optimizing media to improve lactic acid production (Chauhan et al. 2006; Mel et al. 2008). Methods such as factorial design and response surface methodology provide powerful and efficient ways to optimize cultivation and procedures using a reduced number of experiments (Mandenius and Brundin 2008).

The quality of the lactic acid from fermentation media is closely related to recovery and purification methodologies (González et al. 2006). Typically, lactic acid for use in polymers, pharmaceuticals, or food derivatives requires further purification steps; however, the inclusion of these steps is responsible for the high cost of lactic acid obtained by fermentation (Timbuntam et al. 2008). Thus, to reduce costs and solve environmental problems, many studies of the recovery and purification of lactic acid from fermentation media have been conducted using different separation techniques (Marinova et al. 2004; González et al. 2006; Timbuntam et al. 2008).

The present study reports on the highly efficient production of D(−) lactic acid by *S. nakayamae* using peanut flour as a nitrogen source to reduce the production cost. Fermentation conditions were optimized through experimental design. Additionally, a new methodology for the recovery and purification of D(−) lactic acid from the fermentation medium by filtration through activated carbon and Celite, followed by cation exchange chromatography is presented.

Materials and Methods

Bacteria strain and culture conditions

No specific permissions were required for these locations because this microorganism was collected within the university. *S. nakayamae*, a homofermentative lactic acid bacterium that produces D(−) lactic acid with high optical purity, was used in this study. This microorganism was isolated from the rhizosphere of *Desmodium adscendens* collected in the state of São Paulo (Brazil). The strain was stored in GYP medium with 20 % (by volume) glycerol at −80 °C. GYP medium is composed of 2 % glucose, 1 % yeast extract, 1 % peptone, 1 % sodium acetate, and 0.5 % (v/v) salt solution, which was comprised of 4 % $MgSO_4 \cdot 7H_2O$, 0.16 % $MnSO_4.4H_2O$, 0.2 % $FeSO_4.7H_2O$ and 0.2 % NaCl; pH 7.0.

Inoculum and shake-flask fermentation

Inoculum was prepared from activated culture grown on GYP medium incubated at 35 ± 1 °C under stationary conditions until the OD_{600} reached 2.5 (approximately 18–20 h). Experiments were performed in 125-mL flasks containing 18 mL of GYP medium at pH 7.0 and inoculated with 2 mL of the seed culture (10 % inoculum). To avoid a pH decrease because of lactic acid production, 50 g/L of $CaCO_3$ was added

to each flask. The temperature was kept at 35 °C. The cultures were incubated at different conditions, as described below. All experiments were carried out in duplicate to verify their reproducibility, and the results are expressed as mean values. The fermentation samples were centrifuged at 7000 × g for 15 minutes. The resulting supernatant was filtered through a 0.22-μm membrane and was then used to determine the concentration of residual sugar and D(−) lactic acid .

Lactic acid production on different carbon and nitrogen sources

Lactic acid production was studied with different substrates as carbon and nitrogen sources based in GYP medium, with incubation for 48 hours at 35 °C under shaker conditions at 150 rpm.

After the sources that induced the highest lactic acid levels were selected, several concentrations of the carbon and nitrogen sources were evaluated using the experimental designs described below.

Experimental designs

Screening of significant nutritional components of lactic acid production using the Plackett-Burman Experimental Design (PBED)

The PBED was employed for screening and identifying the significance of eight parameters for D(−) lactic acid production. The GYP medium was modified to evaluate the influence of different nutritional components on lactic acid production. Each factor was tested at two levels (coded): a high level (+1) and a low level (−1) (Table 1), and four central points were screened

Table 1 Variables and their levels in lactic acid production under a PBED

Variable	Code	Levels		
		Low (−1)	0	High (+1)
Tween80 (ml/L)	X_1	0	0.5	1
$MnSO_4$ (g/L)	X_2	0	0.05	0.1
$FeSO_4$ (g/L)	X_3	0	0.05	0.1
NaCl (g/L)	X_4	0	0.05	0.1
$MgSO_4$ (g/L)	X_5	0	1	2
Peanut flour (g/L)	X_6	40	60	80
Sodium acetate (g/L)	X_7	0	5	10
Commercial sucrose (g/L)	X_8	80	115	150

Samples were incubated at a temperature of 35 ± 1 °C for 48 hours at 100 rpm. A Fractionated Factorial Design (FFD) was performed according the variables that significantly influenced the production of lactic acid

by running 16 experiments. The factors that were significant at the 5 % level *(P < 0.05)* from the regression analysis were considered to have a high impact on lactic acid production.

Fractional factorial designs

Five variables were studied in a 2^{5-1} FFD to estimate their effect on lactic acid production. A total of 19 experiments were carried out, with 16 experiments related to the design structure and three repetitions at the central point. The two extreme levels are denoted by minus one (lower level) and plus one (higher level), as shown in Table 2.

A new approach of GYP salts was evaluated, and it was added to the solution at certain concentrations. Once peanut flour was established as a nitrogen source, the influence of B vitamins on the production of lactic acid was examined. The fermentation was carried out under shaker conditions (100 rpm) at 35 °C for 48 hours. After incubation, the cultures were processed and quantitatively assayed for lactic acid and residual sugar production.

Response Surface Methodology (RSM)

The RSM was applied to evaluate the interaction of variables and then used to find the optimum concentration of the medium components that greatly affect D(−) lactic acid production (Table 3). To study the influence of commercial sucrose, peanut flour, and Tween 80 on the production of lactic acid by *S. nakayamae*, an RSM was used, with four replicates at the center, totaling 18 experiments.

The flasks were incubated for 72 hours at 35 °C under shaker conditions at 100 rpm. Lactic acid and residual sugar were quantified.

Experimental validation

To validate the optimum values selected by the response surface analysis, two conditions were tested. The concentrations utilized were: 110.00 g/L of commercial sucrose, 150.40 of

Table 2 Design matrix for the fractional 2^{5-1} factorial experiments, used to study the influence of 5 factors on *S. nakayamae* D(−) lactic acid production

Variable	Code	Levels		
		Low (−1)	0	High (+1)
Commercial sucrose (g/L)	X_1	80	100	120
Peanut flour (g/L)	X_2	60	80	100
Tween 80 (ml/L)	X_3	0.5	1	1.5
GYP Salts solution (g/L)	X_4	0	2.5	5
Vitamins (ml/L)	X_5	0	5	10

Table 3 Variables and their levels of lactic acid production under an RSM

Variable		Levels				
		- 1,68	−1	0	+1	+1,68
Commercial sucrose (g/L)	X_1	76,40	90	110	130	143,60
Peanut Flour (g/L)	X_2	49,60	70	100	130	150,40
Tween 80 (ml/L)	X_3	0,16	0,5	1,00	1,5	1,84

peanut flour, and two different concentrations of Tween 80, 1.00 ml/L and 0.16 ml/L.

The fermentation was carried out under shaker conditions (100 rpm) at 35 °C for 72 hours. Each condition was tested five times, and the results are expressed as the mean values.

Purification and recovery of D(−) lactic acid

The fermentation medium was centrifuged at 7000 × *g* at 20 °C for 10 min, and the pH was adjusted to 5.0. Then, 60 ml of the medium (88.27 g/L D(−) lactic acid and 13.50 g/L of sugar) was vacuum filtered through a sintered plate funnel (3.50 cm diameter and 5.00 cm high) containing 1 cm of activated carbon and 1 cm of Celite. The filtrate was filtered through activated carbon and Celite twice, then passed through a cation exchange column (Ambertile IRA 120 hydrogen forms), and filtered through a 0.22-μm membrane. Then, the final solution was used to determine the lactic acid, sugar, and protein concentrations. The total protein concentration was determined by the Lowry method (Peterson 1977).

The lactic acid recovery yield was calculated as follow: lactic acid recovery yield (%) = (final mass (g) / initial mass (g)) × 100.

The sugar removal was calculated using this formula: sugar removal (%) = ((initial mass (g) - final mass (g))/ initial mass (g)) × 100.

Analytical techniques

The concentrations of sucrose, lactose, and lactic acid were determined by high-performance liquid chromatography (HPLC) using a column Rezex ROA 300 mm × 7.8 m (Phenomenex, USA) and a differential refracting index detector (Shimadzu Ultra Fast Liquid Chromatograph, Japan). The mobile phase (0.005 M H_2SO_4) was fed at a flow rate of 0.6 mL/min, and the temperature was kept at 65 °C.

In the end of the optimization process of D(−) lactic acid production, the optical purity of lactic acid produced was determined by HPLC using a Chirex 3126 phenomenex (150 × 4.6 mm) column with 1 mM of CuSO4 as the mobile phase at 1 mL/min (26 °C).

Statistical analysis

The experimental design and the results analysis in all experimental designs were determined using STATISTICA 7 (StatSoft, Tulsa, USA).

Results and discussion

Influence of carbon sources

D(−) Lactic acid production by *S. nakayamae* under the influence of four inexpensive carbon sources was studied (Table 4).

The carbon source that induced the highest lactic acid production was commercial sucrose, producing 59.50 g/L of lactic acid. *S. nakayamae* was also able to produce lactic acid in the presence of sugarcane juice, yielding 53.80 g/L of lactic acid. In the presence of whey and molasses, low levels of lactic acid were produced compared to those of the cultures grown with commercial sucrose and sugarcane juice. It may be that growing the cultures with molasses produced low levels of lactic acid because of the heavy metals (iron, zinc, copper, manganese) found in this substrate, which may inhibit cell growth and inactivate the enzymes involved in final product conversion. However, in previous studies, it was shown that L(+) lactic acid production by *Enterococcus faecalis* from molasses reached a maximum concentration of 134.9 g/L, with a maximum productivity of 4.3 g/Lh (Wee et al. 2006a, b). In the presence of whey, the low level of D(−) lactic acid production should be caused by the inability of *S. nakayamae* to metabolize the lactose, which is the sugar present in this residue, as reported before by Sanders et al. 2003 when referring to the other species of the genus *Sporolactobacillus*. In contrast, in a previous study, it was reported that 52.5 g/L of lactic acid was produced from whey by *Lactobacillus helveticus* (Ghaly et al. 2004).

Among the sources tested, commercial sucrose was selected. Although sugarcane juice was also capable of inducing lactic acid production, this substrate is less interesting in regards to further purification.

Influence of nitrogen sources

Different sources of nitrogen were also studied: corn-steep liquor, Proflo, peanut flour, soybean flour, and urea. The effects of each of these nitrogen sources on lactic acid production are shown in Table 5.

The nitrogen source that induced the highest lactic acid production was peanut flour, corresponding to 57.00 g/L of lactic acid. Moreover, Proflo, induced the production of 52.2 g/L of D(−) lactic acid, and a yield of 0.70 g of lactic acid/g of Proflo. Thus, it was the second most efficient nitrogen source in the production of lactic acid by *S. nakayamae*. This source presents some vitamins on composition, which possibly have a role on this fermentation. Peanut flour and soybean flour have several minerals in their composition (calcium, iron, mMagnesium, manganese, and sodium), which can act as enzyme cofactors. Similar to this study, it has been previously shown that *Bacillus* sp. and *Sporolactobacillus* sp. CASD produce high levels of L(+) and D(−) lactic acid, respectively, using peanut flour as their sole nitrogen source (Meng et al. 2012; Wang et al. 2011).

Moreover, in another previous study, it was reported that *Lactobacillus* sp. produced 41.42 g/L of D(−) lactic acid using corn step liquor and yeast autolysate (Lima et al. 2009). In the present study, when only corn step liquor was used as a nitrogen source, the lactic acid production was not as efficient, yielding 13.26 g/L of D(−) lactic acid . Furthermore, it left a high concentration of residual sugar (70.00 g/L).

Because the nitrogen source accounts for approximately 38 % of the total process cost during lactic acid production (Altaf et al. 2007), the use of wastes and inexpensive nitrogen sources in fermentation medium would reduce these costs.

Experimental designs

Plackett-Burman Experimental Design

The PBED is an efficient screening method for identifying the active factors of an experiment using as few experimental runs as possible.

Table 4 Influence of carbon sources on *S. nakayamae* lactic acid production, productivity, and yield

Carbon sources	Carbohydrates	D(−) Lactic acid Production (g/L)	Productivity (g/Lh)	Yield Yp/s (g/g)	Residual sugar (g/L)
Molasses	46,9 (g/100 mL)[a]	1.06	0.02	0.12	91.40
Commercial sucrose	100 (g/100 g)[a]	59.50	1.23	0.69	14.80
Sugarcane juice	19.95 (g/100 mL)[a]	53.80	1,12	0.66	19.20
Whey	76.59 (g/100 g)[b]	1.26	0.02	0.11	88.57

Culture conditions: GYP medium with 100 g/L of carbon source, 5 % of $CaCO_3$, at 35 °C under shaker conditions at 150 rpm, for 48 h, with an initial pH of 7.0. Type of carbohydrate: a)sucrose; b) lactose

Table 5 Influence of nitrogen sources on *S. nakayamae* lactic acid production, productivity, and yield

Nitrogen sources	N %	D(−) Lactic acid Production (g/L)	Productivity (g/Lh)	Yield Yp/s (g/g)	Residual sugar (g/L)
Proflo	11.15	52.20	1.09	0.71	26.43
Corn step liquor	4,69	13.26	0.28	0.44	70.00
Urea	46.62	38.40	0.80	0.78	51.00
Soybean flour	6.40	41.28	0.86	0.60	30.81
Peanut flour	7.63	57.00	1.19	0.72	21.2

Culture conditions: GYP medium with 100 g/L of commercial sucrose, 5 % of $CaCO_3$, at 35 °C under shaker conditions at 150 rpm, for 48 h, at an initial of pH 7.0. The nitrogen content of all media was 15.25 % ,

The matrix (coded values) of the 16 experiments with eight variables and the respective results are shown in Table 6. After 48 hours of fermentations, the maximum level of lactic acid production was 58.90 g/L (run 7).

The Pareto graph implied that the variables $MgSO_4$, peanut flour, Tween 80, and commercial sucrose influenced the fermentation process significantly at a 95 % confidence level. $MgSO_4$ and commercial sucrose had negative coefficients, while peanut flour and Tween 80 showed positive coefficients (Fig. 1a).

Kitouni and Oulmi (2013) using PBED reported that the use of manganese, magnesium, and iron sulfates as sources of oligoelements appears to have no significant effect on the lactic acid production by *Lactobacillus bulgaricus* attc 11842 grown on whey.

Once the peanut flour presents high lipid concentration in the composition (51.77 g/100 g), the surfactant property of

Tween 80 can interact with lipids, improving the solubility of the nutrients found in peanut flour. Tween 80 may assist in nutrient absorption by microorganisms, as well as product liberation by facilitating transport via the cell membrane of the microorganism. Conversely, Coelho et al. 2011a, b reported that Tween 80 had a significant positive effect on lactic acid production by *Lactobacillus plantarum* LMISM6.

The negative effect of commercial sucrose is probably related to the high concentration used, which could inhibit the lactic acid production. Therefore, the components selected for the next experiment were peanut flour, commercial sucrose, and Tween 80.

Several studies reported the use of PBED successfully employed to screen the significance components in culture media, in order to improve the lactic acid production (Wang et al. 2015; Qin et al. 2009; Chauhan et al. 2007). Gowdhaman et al. (2012) using PBED was able to select three variables

Table 6 PBED with coded values and experimental results for lactic acid production, productivity, yield, and residual sugar concentration

Run	Independent variables								Lactic acid g/L	Productivity g/L/h	Yield Y p/s g/g	Residual sugar g/L
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8				
1	1	−1	1	−1	−1	−1	1	1	52.44	1.09	0.80	84.35
2	1	1	−1	1	−1	−1	−1	1	44.40	0.92	0.69	86.02
3	−1	1	1	−1	1	−1	−1	−1	30.43	0.63	0.99	49.15
4	1	−1	1	1	−1	1	−1	−1	55.85	1.16	0.98	22.92
5	1	1	−1	1	1	−1	1	−1	51.05	1.06	0.97	27.63
6	1	1	1	−1	1	1	−1	1	45.78	0.95	0.78	91.09
7	−1	1	1	1	−1	1	1	−1	58.90	1.22	0.99	23.75
8	−1	−1	1	1	1	−1	1	1	15.64	0.32	0.76	133.72
9	−1	−1	−1	1	1	1	−1	1	40.96	0.85	0.77	96.73
10	1	−1	−1	−1	1	1	1	−1	54.73	1.14	0.98	24.42
11	−1	1	−1	−1	−1	1	1	1	48.70	1.01	0.40	98.93
12	−1	−1	−1	−1	−1	−1	−1	−1	51.73	1.08	0.95	25.42
13	0	0	0	0	0	0	0	0	51.19	1.07	0.96	61.61
14	0	0	0	0	0	0	0	0	46.38	0.97	0.76	53.70
15	0	0	0	0	0	0	0	0	50.25	1.05	0.78	50.60
16	0	0	0	0	0	0	0	0	51.59	1.07	0.94	59.97

X_1 = Tween80. X_2 = $MnSO_4$. X_3 = $FeSO_4$. X_4 = NaCl. X_5 = $MgSO_4$. X_6 = Peanut flour. X_7 = Sodium acetate. X_8 = Commercial sucrose. Culture conditions: 35 °C, initial pH of 7.0 neutralized by 5 % $CaCO_3$, 100 rpm of agitation, 10 % inoculum. Fermentation was carried out for 48 hours
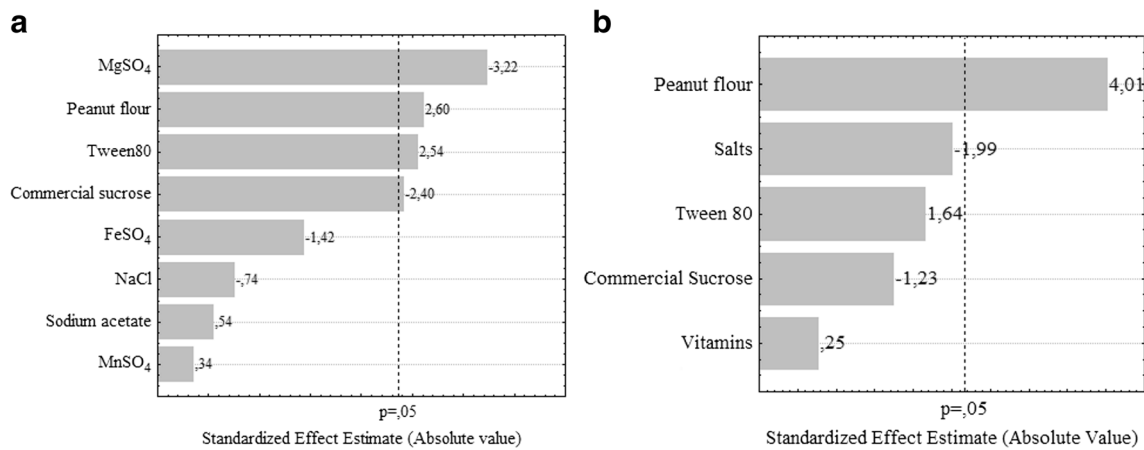
Fig. 1 Pareto graph for D(−) lactic acid production. **a**) PBED; **b**) FFD

namely substrate concentration, moisture content, and yeast concentration that have statistically significant effect on lactic acid yield by *Lactobacillus plantarum* MTCC 6161 in solid-state fermentation. Chauhan et al. 2006, studying optimization of lactic acid production using date juice, reported that among the fifteen variables (date juice, peptone, beef extract, yeast extract, K2HPO4, KH2PO4, MgSO4 · 7H2O, MnSO4 · H2O, sodium acetate, sodium sulfate, tri-sodium citrate, sodium succinate, tween-80, F,eSO4 and NaCl), significant components were screened out using PBED . This experiment

suggested four components, date juice, peptone, K2HPO4, and sodium acetate, as having significant effects on lactic acid production by *Lactobacillus* sp. KCP01.

Fractional factorial designs (FFD)

In this study, a 2^{5-2} FFD was used to determine the significant factors of five media components. The 19 runs and the results of the response FFD are presented in Table 7.

Table 7 Fractional factorial design with coded and experimental results for lactic acid production, productivity, yield, and residual sugar concentration

Run	Independent variables					Lactic acid	Productivity	Yield Yp/s	Residual sugar
	X_1	X_2	X_3	X_4	X_5	g/L	g/Lh	g/g	g/L
1	−1	−1	−1	−1	1	48.49	1.01	1.20	39.70
2	1	−1	−1	−1	−1	41.76	0.87	0.48	33.15
3	−1	1	−1	−1	−1	47.82	1.00	0.67	8.70
4	1	1	−1	−1	1	46.46	0.97	0.70	53.74
5	−1	−1	1	−1	−1	46.24	0.96	0.62	5.35
6	1	−1	1	−1	1	44.35	0.92	0.85	67.86
7	−1	1	1	−1	1	49.02	1.02	0.97	29.21
8	1	1	1	−1	−1	51.92	1.08	0.87	60.04
9	−1	−1	−1	1	−1	42.79	0.89	0.99	36.85
10	1	−1	−1	1	1	43.05	0.90	0.80	66.22
11	−1	1	−1	1	1	46.87	0.98	1.04	35.08
12	1	1	−1	1	−1	44.01	0.92	0.83	66.79
13	−1	−1	1	1	1	42.16	0.88	1.01	38.08
14	1	−1	1	1	−1	42.79	0.89	0.80	66.63
15	−1	1	1	1	−1	49.59	1.03	0.98	29.53
16	1	1	1	1	1	48.59	1.01	0.98	70.22
17	0	0	0	0	0	43.15	0.90	0.85	49.41
18	0	0	0	0	0	44.205	0.92	0.89	50.13
19	0	0	0	0	0	44.465	0.93	0.86	48.52

X_1 = Commercial sucrose X_2 = Peanut flour. X_3 = Tween 80. X_4 = Salt solution. X_5 = Vitamins. Culture conditions: 35 °C, initial pH 7.0 neutralized by 5 % CaCO$_3$, 100 rpm of agitation, 10 % inoculum. Fermentation was carried out for 48 hours

Table 8 RSM design with coded values and experimental results for lactic acid production, productivity, yield, and residual sugar concentration

Run	Independent variables			Lactic acid	Productivity	Yield Y p/s	Residual sugar
	X_1	X_2	X_3	g/L	g/Lh	g/g	g/L
1	−1	−1	−1	78.10	1.08	0.88	15.20
2	1	−1	−1	85.48	1.19	0.89	54.97
3	−1	1	−1	81.67	1.13	0.79	9.95
4	1	1	−1	99.46	1.38	0.85	22.85
5	−1	−1	1	83.77	1.16	0.92	9.89
6	1	−1	1	96.18	1.34	0.86	28.32
7	−1	1	1	78.81	1.09	0.91	6.80
8	1	1	1	101.99	1.42	0.91	26.13
9	−1,68	0	0	82.40	1.14	0.95	5.39
10	1,68	0	0	88.68	1.23	0.79	41.50
11	0	−1,68	0	88.53	1.23	0.91	23.80
12	0	1,68	0	99.99	1.39	0.86	10.24
13	0	0	−1,68	93.47	1.30	0.84	16.24
14	0	0	1,68	93.11	1.29	0.84	17.92
15	0	0	0	93.53	1.30	0.88	20.88
16	0	0	0	91.60	1.27	0.84	17.80
17	0	0	0	88.80	1.23	0.85	22.66
18	0	0	0	92.02	1.28	0.85	19.12

X1 = Commercial sucrose, X2 = Peanut flour, X3 Tween 80. Culture conditions: 35 °C, Initial pH 7.0 neutralized by 5 % CaCO₃, 100 rpm of agitation, 10 % inoculum. Fermentation was carried out for 72 hours

The highest lactic acid concentration was identified on run 8 (52.92 g/L). This run contained the highest concentrations of carbon and nitrogen sources and contained no salts or vitamins; however, the level of residual sugar found in this experiment was high (60.04 g/L).

After 48 hours of fermentation, the peanut flour had a positive and significant effect on D(−) lactic acid production (Fig. 1b).

The commercial sucrose and salts, despite being presented as not significantly influential, had negative effects on lactic acid production. This negative effect may be associated with the amount of carbohydrates and mineral salts that already exist in peanut flour, causing a high concentration of these components on the culture media. As previously mentioned, the negative effect of commercial sucrose is related to product inhibition mediated by high substrate concentration. Different
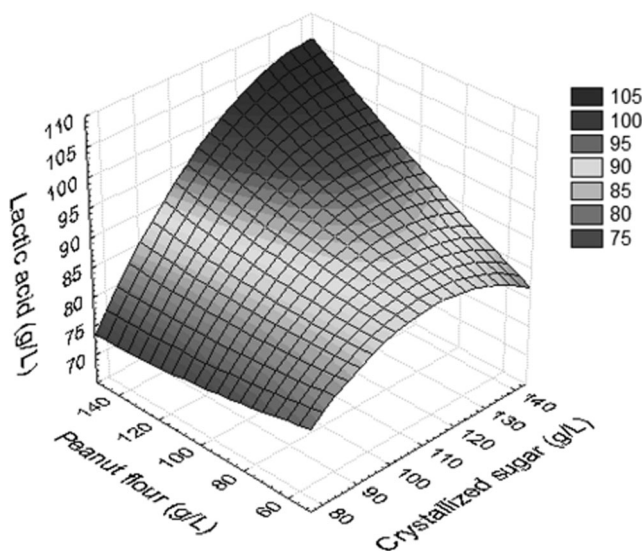

Fig. 2 Response surface for lactic acid production by *S. nakayamae* showing the interaction between peanut flour and commercial sucrose
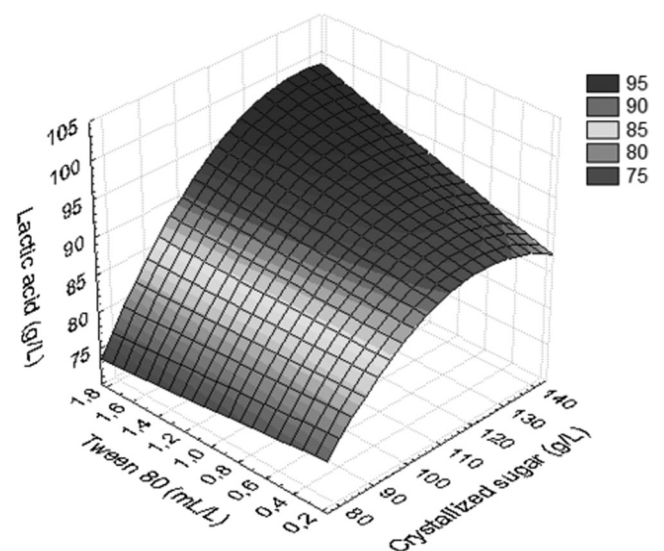

Fig. 3 Response surface for lactic acid production by *S. nakayamae* showing the interaction between Tween 80 and commercial sucrose
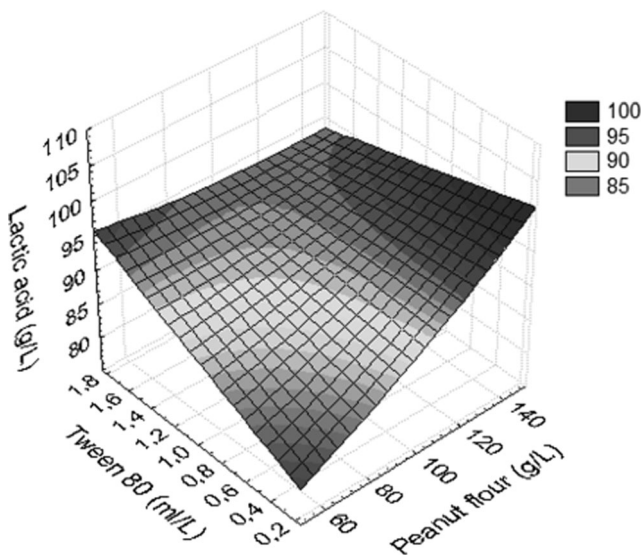
Fig. 4 Response surface for lactic acid production by *S. nakayamae* showing the interaction between Tween 80 and peanut flour

results were reported by Vasala et al. (2005), who showed that *Lactobacillus salivarius* ssp. is an ideal organism for lactic acid fermentation in high-salt high-lactose conditions. Additionally, the vitamins and Tween 80 did not significantly influence lactic acid production; nevertheless, it was verified that when the highest concentration of Tween 80 was used, there was higher lactic acid production. However, previous studies reported a positive influence of B vitamins on lactic acid production (Yoo et al. 1997; Altaf et al. 2007).

Considering these results, Tween 80, commercial sucrose, and peanut flour were selected for use in the RSM.

Response Surface Methodology (RSM)

Three factors, commercial sucrose, peanut flour, and Tween 80, were distributed in three levels, and two axial points and four central points were evaluated. The responses measured were D(−) lactic acid concentration, productivity, yield, and residual sugar after 72 h of fermentation (Table 8). Among the concentration of nutrients evaluated, 130 g/L of commercial sucrose, 130 g/L of peanut flour, and 1.5 ml/L of Tween 80 (run 8) achieved the highest D(−) lactic acid production

(101.99 g/L) and productivity (1.42 g/L h), although there was a high concentration of residual sugar (26.13 g/L).

The results obtained were fitted to multiple regression analysis to yield the following regression equation:

$$Y = 91.62 + 5.22X_1 + 2.75X_2 + 1.12X_3 - 2.71X_{1^2}$$
$$+ 2.64X_1X_2 + 1.30X_1X_3 + 0.37X_{2^2} - 2.08X_2X_3$$
$$+ 0.02X_{3^2}$$

in which Y is the predicted response (D(−) lactic acid production) and X1, X2, and X3 are the coded values of the test variables for commercial sucrose, peanut flour, and Tween 80, respectively.

An ANOVA of the quadratic regression model demonstrated that the model is significant, as determined by Fisher's test (Fcalc (3.81) > Ft (3.39)). It was also determined that the model has a very low probability value ((Pmodel > F) =0.03). The fit of the model was checked by the coefficient of determination (R^2) and multiple correlation coefficients (R). The R^2 value (0.81) for the regression equation indicated that the sample variation for lactic acid of 81 % was attributed to the independent variables. In this case, the value of the coefficient of determination indicated that only 19 % of the total variations were not explained by the model. The high R value (0.90) demonstrated a strong agreement between observed and predicted values. Among the model terms, X1 was significant with a probability of 95 %.

The response for the regression equation was plotted in Figs. 2, 3, and 4. The following graphs show the interaction of the variables and the optimum levels of each variable for D(−) lactic acid production.

Based upon the response surfaces and the values presented in Table 8, run 12 was considered the most appropriate combination for D(−) lactic acid production. This run consisted of 110 g/L of commercial sucrose, 150.40 g/L of peanut flour, and 1 mL/L of Tween 80, producing 99.99 g/L of D(−) lactic acid, with a 1.39 g/Lh rate of productivity and a low concentration of residual sugar (10.24 g/L).

When the highest levels of commercial sucrose and peanut flour were used, it was verified that this condition produced high levels of lactic acid; however, the amount of residual sugar produced was substantial. The same results were verified on

Table 9 Purification parameters of the fermentation media

Parameters	Lactic acid	Sugar	Protein
[Initial solution] (g/L)*	88.27	13.50	13.54
Initial mass (g)	5.296	0.810	0.812
[Final solution] (g/L)**	16.85	0.06	0.00
Final mass (g)	4.212	0.015	0.000
Index	Recovery Yield 79 %	sugar removal 98 %	protein removal 100 %

* Initial volume 60 mL; ** Final volume 250 mL

commercial sucrose and Tween 80 interaction. The response surface between peanut flour and Tween 80 showed that intermediate concentrations of this components did not produce a satisfactory amount of D(−) lactic acid . Additionally, it was found that low Tween 80 concentrations combined with high peanut flour concentrations showed higher levels of D(−) lactic acid production.

Previous studies have reported that RSM is an efficient method for the determination of optimized culture conditions for the production of lactic acid (Lima et al. 2011; Coelho et al. 2011a, b). Chaisu et al. (2014) reported, using RSM, the best percentage of inoculum (8.02 %) and carbon source (3.82 % of molasses) to lactic acid production (38.33 %) by *Lactobacillus casei* M-15 within 24 hr at 37 °C. Bustos et al. 2004 demonstrated the optimization of culture media applying RSM using *Lactobacillus coryniformis.* The models predicted a maximum D(−) lactic acid concentration (58.9 g/L) at 96 h using 5 g corn steep liquor/L, 3.6 g Yiest Extract/L, and 10 g Peptone/L. In turn, Lima et al. (2010) through RSM verified that the best results for lactic acid production (52.37 g/L) were obtained with 59.64 g/L of lactose, 14.55 g/L of corn steep liquor, and 5.65 g/L of (NH4)2SO4, at 39.6 °C by *Lactobacillus* sp. LMI8.

Experimental validation

A low concentration of Tween 80 (0.16 mL/L) combined with 110 g/L of commercial sucrose and 150.40 g/L of peanut flour induced a higher D(−) lactic acid production (112.93 g/L), productivity (1.57 g/L.h), and yield (0.98 g/g) with a reduced residual sucrose concentration (9.6 g/L). Compared with conditions utilizing a higher concentration of Tween 80 (1.00 mL/L), low levels of D(−) lactic acid (104,38 g/L), productivity (1, 45 g/L.h) and yield (0,96 g/g) were presented.

In order to verify the optical purity of lactic acid produced, the optimized culture media was analyzed by HPLC using a chiral column. *S. nakayamae* produced 98.75 % of D(−) lactic acid and 1.24 % of L(+) lactic acid, thus it is considered a homofermentative microorganism.

Purification and recovery of D(−) lactic acid

The centrifuged fermentation medium at pH 5.0 was submitted to the purification process, which involved two steps: (1) double filtration on activated carbon and Celite, and (2) the transformation of calcium lactate into lactic acid via cation resin exchange. The purification and recovery assays were performed in duplicate, and the results are expressed as the mean values (Table 9).

D(−) lactic acid was efficiently recovered from fermentation medium (79 %). Additionally, protein and sugar were efficiently removed (100 % and 98 %, respectively). Previous studies have reported similar recovery yields using

other purification techniques, such as solvent extraction, multiple-pass distillation, and micellar-enhanced ultrafiltration (Chen et al. 2012; Geanta et al. 2013). Furthermore, it was verified that there was a low ratio of sugar to lactic acid (0.36 %), which is essential to polymer synthesis for avoiding caramelization.

Conclusions

In this study, it was found that *S. nakayamae* is able to produce high levels of D(−) lactic acid using peanut flour and commercial sucrose as substrates. Experimental design methodology effectively determined the significant nutrients and optimum concentration of medium components. The purification methodology works with inexpensive and reusable reagents. In addition, the high performance of this method of D(−) lactic acid production utilizing cheap materials will be beneficial for bioplastic technologies. However, to be applied on a large scale in the lactic acid industries, additional well-established research efforts need to conducted.

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