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Optimization of Nisin Production by *Lactococcus lactis*

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Abstract

The production of nisin by batch culture of *Lactococcus lactis* ATCC 11454 in MRS broth (pH 6.5), as treated in 30 assays, that were set up by a fractional factorial design of two levels ($2^{[4-1]}$), was improved. The minimum and maximum concentrations of sucrose (5.0–12.5 g/L), asparagine (7.5–75 g/L), potassium phosphate (6.0–18.0 g/L), and Tween-80 (1.0–6.6 g/L) were added to MRS broth. The best nisin activities ranged from 1.5×10^4 to 1.8×10^4 arbitrary units (AU)/mL for the maximum levels of sucrose, asparagine, and monobasic potassium phosphate, and for the minimum concentration of Tween-80. The best following proportions between nutrients were adopted as optimum for maximum specific nisin productivity of about 6.0 mg/mg of dry cell weight (related to 2.5 mg of pure nisin preparation with a specific activity of 1.0×10^5 AU/mL): C/N = 0.17, C/P = 0.69, N/P = 4.17 (C = sucrose, N = asparagine, P = phosphate, T = Tween-80).

Index Entries: Nisin; nutrient balance; *Lactococcus lactis*; *Lactobacillus sake*; batch fermentation.

Introduction

Nisin is a type A bacteriocin, whose chemical structure was originally described more than 30 yr ago *(1)*, and its bacterial inhibitory activity has been known for more than 50 yr *(2)*. There are two naturally occurring nisin variants, nisin A and nisin Z, which differ in a single amino acid residue at position 27. Asparagine in nisin Z is replaced by histidine in nisin A *(3,4)*. Nisin Z appears to be slightly more diffusable in agar and more soluble in neutral pH. The term *nisin* designates the mature, active bacteriocin, a

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monomeric pentacyclic molecule (3353 Daltons), soluble at pH 2.0 and insoluble at pH 8.5 *(5)*.

Nisin is a bacteriocin produced by *Lactococcus lactis* subspecies *lactis* during the exponential phase of growth of the microorganism *(6,7)*. Nisin strongly inhibits the outgrowth of spores and the growth of a broad range of Gram-positive microorganisms. The growth of a variety of Gram-negative bacteria can also be inhibited by nisin if the outer membrane is first destabilized by EDTA *(8)*.

The effect of nisin on the targeted bacteria is exerted in the cytoplasic membrane. The main activity appears to be the formation of pores in the bacterial membrane. Pore formation causes an increase in membrane permeability with a concomitant leakage of ions, adenosine triphosphate, and amino acids, which, in turn, leads to the dissipation of the bacterial membrane potential. The combined effect of energy depletion and the efflux of essential compounds inhibits synthesis of macromolecules and results in an ultimate cell death accompanied, in most cases, by cell lysis. Nisin action against spores is caused by binding protein residues with sulfydryl groups on the spore coats with resulting mechanical rupture *(9)*. Because of the broad spectrum of antibacterial activity, nisin is used as a preservative in food industries and as a potential therapeutic agent. It can be successfully used to increase the shelf life of processed cheese, dairy desserts, milk, canned food, meat, fish, and alcoholic beverages *(10)*. Nisin was approved by the Food and Drug Administration in 1988 as Generally Recognized as Safe *(5)*, meeting the demands for natural foods with fewer chemical additives. The most promising and the most important group of natural inhibitors is the bacteriocins, with a highly economic added value. Therefore, the relevant nutritional factors for stimulating the growth of lactobacillus and their simultaneous production of bacteriocins have been investigated.

Lactic acid bacteria are fastidious microorganisms and require a medium containing nutrients, that enhance the growth and production of bacteriocin *(11,12)*. Several researchers have attempted to develop an optimal medium for maximum production of bacteriocin, which is directly related to the growth of lactobacilli *(5,12)*. In addition, it was verified that production stops completely when cells enter the stationary phase *(7)*. The synthetic Lactobacilli MRS medium, which are less complex than dairy products, and consequently more reliable for reproducibility assays, have been used to enable us to study the influence of added chemical components and their associations on the production of nisin simultaneously with the optimum growth of *L. lactis*.

It was verified that nisin yield was dependent on the concentrations of different sugars (glucose, sucrose) in the culture medium *(13)*. It was further verified that xylose was a very efficient carbon source for nisin production in the batch process with immobilized *L. lactis* in calcium alginate beads *(14)*. Moreover, sucrose, although a disaccharide, is rapidly used by nisin-producing *L. lactis* subspecies *lactis* strains. Generally, the higher the initial concentration of sucrose, the greater the increase in nisin production

at the end of the exponential phase, and the lower the decrease in nisin titer during prolonged fermentation. A genetic linkage exists between the sucrose fermentation capacity and the ability to produce nisin *(7)*.

Elli et al. *(12)* studied the influence of growth factors on the fermentation of the probiotic strain *Lactococcus johnsonii* NCC 533 in milk. They verified that the supplementation of milk with four amino acids (DL-alanine, DL-serine, DL-isoleucine, and L-cysteine), four nucleosides (adenosine, guanosine, cytidine, and uridine), and one source of iron (ferrous sulfate) strongly stimulated its growth. However, the *L. johnsonii* could grow in the absence of those exogenous chemical substances. Lactobacilli's growth can also be improved by the addition of substances of undefined composition such as yeast extract or peptones *(15)*. Their positive influence could be attributed to their nucleotide and amino acid contents. However, Ellis et al. *(12)* verified that the amount of yeast extract as a medium supplement was dependent on different suppliers.

It was further verified that asparagine is an interesting source of nitrogen since it is easily assimilated, inexpensive, and of recognized purity, as distinct from the conventional sources of nitrogen, such as peptones and yeast or meat extract, in which the composition of nitrogen or protein varies from lot to lot in industrial production.

The effect of lowering the pH on cellular growth is partially owing to the concentration of dissociated and nondissociated forms of lactic acid in the broth. To avoid growth inhibition by the decrease in pH resulting from the excess of lactic acid in solution, Shimizu et al. *(16)* worked with lactate removal by mixed cultures. It was verified that supplementation of the medium with a buffering system composed of acetate, citrate, and phosphate, for a pH ranging from 5.9 to 6.1, improved the growth of *L. lactis*, as well as increased the yield of nisin *(5,7)*. The presence of potassium dihydrogen phosphate was suggested to be essential in the formulation of a synthetic complex medium owing to its buffering agents characteristics, as well as to be a source of potassium ions, which are necessary for cell development.

Tween-80, a constituent in the Lactobacilli MRS medium, is commonly used as a dispersing agent in medium composition *(17,18)*. Furthermore, nisin tends to remain adsorbed in the cellular membrane owing to its amphipathic nature. Therefore, dispersing agents might be of interest for the promotion of its release from the cell membrane to the culture medium and consequent increase in productivity, since it was verified that nisin accumulation in membranes regulated prenisin synthesis in a gene cluster *(19)*.

The purpose of the present study was to optimize the growth parameters of *L. lactis* ATCC 11454 in order to improve its expression of nisin, in a batch fermentation process at uncontrolled pH. The optimization of the growth medium was investigated with the addition of sucrose as the carbon source, asparagine as the nitrogen source, potassium phosphate as the buffering agent and Tween-80 as the dispersing agent.

Material and Methods

Cultures and Inocula

The bacteria used were Lactococcus lactis (ATCC 11454) as the nisin-producing strain, and *Lactobacillus sake* (ATCC 15521) as the sensitive bacteria for the activity test of the expressed nisin, both stored at –80°C in the Lactobacilli MRS (Difco, Detroit, MI) broth supplemented with 40% glycerol. One hundred microliters of the frozen strains was inoculated into 50 mL of the MRS broth and grown in a rotary shaker (100 rpm) (model SR 12; Fanem, Brazil) for 24 h, at 30°C for the inoculum's preparation. The broth culture with *L. sake* was added to the 50 mL of Lactobacilli MRS (Difco) agar for the detection of nisin by means of the agar diffusion method. Five milliliters of the L. lactis inocula (optical density at 600 nm [OD_{600nm}] = 0.7), previously prepared, was homogenized in 50 mL of the MRS broth. After being supplemented with the nutrients, flasks were shaken in a rotatory shaker (100 rpm at 30°C for 36 h) with 50 mL of the supplemented medium and tests were then conducted.

The medium's constitution for complex Lactobacilli MRS (Difco) broth (10.0 g/L of peptone, 10.0 g/L of beef extract, 5.0 g/L of yeast extract, 20.0 g/L of dextrose, 1.0 g/L of Tween-80, 2.0 g/L of ammonium citrate, 5.0 g/L of sodium acetate 0.1 g/L of magnesium sulfate, 0.05 g/L of manganese sulfate, 2.0 g/L of dibasic potassium phosphate) was supplemented with a carbon source, sucrose (Merck); a nitrogen source, asparagine (Merck); a buffering agent, monobasic potassium phosphate (Merck); and a dispersing agent, Tween-80 (Merck), from assays 1 to 8, group 1 (Table 1).

Experimental Design

The addition of the nutrients to the complex MRS medium was set up by a fractional factorial $2^{(4-1)}$ design, at two levels (–1 to +1), considering group 1 (assays 1–8) and group 2 (assays 9–24), shown in Table 1. The model was later extended to six central points, when the concentrations of sucrose, asparagine, and monobasic potassium phosphate were codified in the limits of (–1.68) to (+1.68), corresponding to assays 25–30. The maximum and minimum levels of concentrations employed in the assays of groups 1, 2 and 3, respectively, ranged as follows: for sucrose, from 2.0 to 5.0, from 5.0 to 12.5, and from 2.25 to 15 (g/L); for asparagine, from 1.5 to 15, from 7.5 to 75, and from 7.5 to 98 g/L; for potassium phosphate, from 3.0 to 6.0, from 6.0 to 18.0, and from 6.0 to 22.0 g/L, as shown in Table 1. The supplemented concentration of Tween-80 added to the concentration (1.0 g/L) in the MRS medium in assays 1–11 varied between 2.1 and 6.6 g/L. For the other assays, the concentration of 1.0 g/L of Tween-80 was considered. For every batch fermentation assay, at uncontrolled pH, 12 Erlenmeyer flasks (250 mL) containing 50 mL of the supplemented MRS medium each were placed in a rotary shaker at a with controlled temperature of 30°C for 36 h (assays 1–11) and 48 h (assays 12–30). The pH measurements were carried out in a potentiometer (model B 12; Procyon, Brazil).

Table 1
Nutrients and Their Proportions Added to MRS Medium

Nutrients Added						Proportion			
Group	Assay	Sucrose (g/L)	Asparagine (g/L)	Phosphate (g/L)	Tween-80[a] (g/L)	C/N[b]	C/P[c]	N/P[d]	T/P[e]
1	1	2	1.5	3	2.1	1.33	0.67	0.50	0.70
1	2	5	1.5	3	6.6	3.33	1.67	0.50	2.20
1	3	2	15	3	6.6	0.13	0.67	5.00	2.20
1	4	5	15	3	2.1	0.33	1.67	5.00	0.70
1	5	2	1.5	6	6.6	1.33	0.33	0.25	1.10
1	6	5	1.5	6	2.1	3.33	0.83	0.25	0.35
1	7	2	15	6	2.1	0.13	0.33	2.50	0.35
1	8	5	15	6	6.6	0.33	0.83	2.50	1.10
2	9	12.5	75	18	6.6	0.17	0.69	4.17	0.37
2	10	12.5	75	6	2.1	0.17	2.08	12.50	0.35
2	11	12.5	75	18	2.1	0.17	0.69	4.17	0.12
2	12	5	7.5	6	1.0	0.67	0.83	1.25	0.17
2	13	12.5	7.5	6	1.0	1.67	2.08	1.25	0.17
2	14	5	75	6	1.0	0.07	0.83	12.50	0.17
2	15	12.5	75	6	1.0	0.17	2.08	12.50	0.17
2	16	5	7.5	18	1.0	0.67	0.28	0.42	0.06
2	17	12.5	7.5	18	1.0	1.67	0.69	0.42	0.06
2	18	5	75	18	1.0	0.07	0.28	4.17	0.06
2	19	12.5	75	18	1.0	0.17	0.69	4.17	0.06
2	20	8.75	41.25	12	1.0	0.21	0.73	3.44	0.08
2	21	8.75	41.25	12	1.0	0.21	0.73	3.44	0.08
2	22	8.75	41.25	12	1.0	0.21	0.73	3.44	0.08
2	23	8.75	41.25	12	1.0	0.21	0.73	3.44	0.08
2	24	8.75	41.25	12	1.0	0.21	0.73	3.44	0.08
3	25	2.25	41.25	12	1.0	0.05	0.18	3.44	0.08
3	26	15	41.25	12	1.0	0.36	1.25	3.44	0.08
3	27	8.75	7.5	12	1.0	1.17	0.73	0.63	0.08
3	28	8.75	98	12	1.0	0.09	0.73	8.17	0.08
3	29	8.75	41.25	6	1.0	0.21	1.46	6.88	0.17
3	30	8.75	41.25	22	1.0	0.21	0.40	1.88	0.05

[a]Tween-80, added to the original medium concentration (MRS broth).
[b]C/N = sucrose/asparagine relation (w/w).
[c]C/P = sucrose/phosphate relation (w/w).
[d]N/P = asparagine/phosphate relation (w/w).
[e]T/P = Tween-80/phosphate relation (w/w).

Assays

The cell biomass concentration, expressed in milligrams of dry cell weight (DCW) per liter of broth, was attained by the OD_{600nm} in a 1.0-cm optical pathway quartz cuvet in a spectrophotometer (Incibras MS UV/VIS). The OD_{600nm} readings were calibrated against a standard dried cell concentration curve of *L. lactis*, which was obtained by the gravimetry method of

the biomass (mg/L) held on the surface of a 0.22-μm membrane (Millipore, SP, Brazil). The equation for the calibration curve ($R^2 = 0.998$) was as follows: DCW (mg/L) = $0.012 + 1.4 \times (OD_{600nm})$.

The final sucrose (g/L) concentrations in the broth were obtained at the end of the assays. The Somogyi-Nelson *(20)* method was used following acid hydrolysis (5 mL of 1.3 *N* HCl for 15 min at 70°C) for the sucrose (total reducing sugars) analysis, and titration with 0.1 *N* HCl previously standardized for the lactic acid titer. The calibration curve ($R^2 = 0.998$), expressing sucrose in total reducing sugars, was as follows: sucrose (mg/L) = $431.97 \times (OD_{540nm}) - 9.08$.

The activity of the expressed nisin was evaluated by the agar diffusion method. Ten milliliters of the inoculated MRS plus 1.5% agar with a 24-h culture ($OD_{600nm} = 0.6$) of *L. sake* were placed on a sterile plate, so that, after solidifying, small wells (approx 3.0 mm in diameter) were cut *(21)*. Fifty-microliter samples from the fermented *L. lactis* broth, previously adjusted to pH 6.5 with 0.1 *N* NaOH, were put into the wells, and the plates were incubated at 30°C for 24 h. Measurement of the diameter of the growth inhibition zone, correlated to a concentration of standard nisin, allowed the nisin concentration in the sample (arbitrary units per milliliter: [AU/mL]) to be estimated by the calibration curve ($R^2 = 0.98$), in which the diameter of the inhibition zone was related to the decimal logarithm of AU/mL: Diameter (cm) = $1.01 + 0.24 \times \log_{10}$ AU/mL. To set up a calibration curve, a solution of Nisaplin (Aplin & Barrett, Sigma, St. Louis, MO) containing 25,000 μg of nisin/g (at activity of 10^6 IU/g) was dissolved in a 0.02 *N* HCl (plus 0.75% [w/v] NaCl) solution serial decimal dilutions with a final pH adjusted to 3.0 prior to steam sterilization (121°C for 15 min), and then adjusted to pH 6.5 and applied to the wells; the inhibition zone was measured after plate incubation (30°C for 24 h). Before each experiment, a stock solution of 250 μg of nisin/mL (10^4 IU/mL) was prepared as a reference standard.

For assays 9–24, interactions among the four nutrients (variables) added to the complex MRS medium on the activity of nisin expressed during *L. lactis* growth were calculated to establish the best combination of nutrients to improve nisin production. The main effects of the variables, interaction coefficients (95%), correlation matrix for estimated parameters, respective confidence intervals, significance levels ($p < 0.05$), and regression variance analysis (analysis of variance) *(22)* were calculated through the SGWIN program (Statgraphics Plus for Windows version 1.4; Statistical Graphics, 1995). The four nutrient variables (x) considered in regression analysis were taken by nondimensionable values over the same (–1) to (+1) range. The maximum (+1) and minimum (–1) codified concentrations for each independent variable were as follows:

x_1 = sucrose (5.0–12.5 g/L); x_2 = asparagine (7.5–75 g/L); x_3 = potassium phosphate (6.0–18.0 g/L); x_4 = Tween-80 (1.0–6.6 g/L).

The intermediate levels in code units were given by the following equation:

$$\text{Codified variable } (x_n) = \frac{(\text{nutrient} + \text{concentration}) - (\text{maximum} + \text{minimum concentration})/2}{(\text{maximum} - \text{minimum concentration})/2}$$

Results and Discussion

Nisin is continuously detected from the early growth of *L. lactis* to the stationary phase. All the genes needed for nisin synthesis are transcribed several hours before nisin can be detected in growth medium *(5,6,7,23)*.

The microbial growth of *L. lactis* and nisin production kinetics (Table 2) were examined in the supplemented MRS broth, with sucrose, asparagine, potassium phosphate, and Tween-80 (Table 1) in a batch fermentation process at uncontrolled pH.

Regarding group 1, for 2 g/L of sucrose, the maximum titer of nisin reached was about 200 AU/mL, independent of phosphate, asparagine, and Tween-80 concentrations (Table 2). By increasing the concentration of sucrose to 5 g/L, the maximum titer of nisin varied from 298 to 7770 AU/mL, the lowest activity of nisin corresponding to minimum concentrations of asparagine and phosphate. For concentrations 10 times greater in relation to asparagine (from 1.5 to 15 g/L), keeping minimum concentrations of phosphate and Tween-80, the nisin titer rose 16 times (4.8×10^3 AU/mL, assay 4), showing asparagine as a limiting factor in the consumption of sucrose. For the maximum concentrations of all components added to the complex MRS medium, the maximum nisin titer reached 7.8×10^3 AU/mL (assay 8), raising nisin productivity from 33.40 to 53.96 mg/(L·h).

Amiali et al. *(18)* have obtained similar production of nisin Z (1.6×10^4 to 2.5×10^4 IU/mL) has been obtained by, using yeast extract/Tween-80-supplemented whey permeates as a complex medium, with aeration control of the process.

A multiple linear regression was applied to the data related to the decimal logarithm of the nisin activity (dependent variable) obtained at the end of *L. lactis* growth periods set for assays 9–24. The regression analysis attained a quadratic polynomial model (for the independent variables x_1 = sucrose, x_2 = asparagine, x_3 = potassium phosphate, and x_4 = Tween-80):

$$\log_{10} \text{AU/mL} = 1.99 - 0.21x_2 + 1.80x_3 - 1.41x_4 + 0.23x_1x_2 + 0.36x_1x_3 + 1.47x_3x_4$$

Main equation is shown in Table 3.

The analysis of the statistical coefficients (annex 1), at the significance level of $p < 0.05$, provided statistical significance to the polynomial models and consistency to the estimated regression coefficients. Therefore, its geometric interpretations, such as surface response graph (Fig. 1), for the first-order equations are reliable, showing the tendency caused by the independent variables. The influences of x_1, x_2, x_3, and x_4 were nonlinear in the decimal logarithm of nisin activity expressed in arbitrary units per milliliter ($\log_{10}$ AU/mL). The individual contributions of phosphate (x_3)

Table 2
Kinetic Parameters for Nisin Production by *L. lactis* in Batch Fermentation Process Under Uncontrolled pH

Assay	Final pH	Sucrose (g/L)	DCW (mg/L)	Nisin (AU/mL)	$\log_{10}$ (AU/mL)	μX^2 (h^{-1})	AU/mg DCW (AU/mg)	Nisin (mg/L)	Nisin (mg/[L·h])	Nisin mg/mg DCW
1	5.65	1.01	557.0	1.3×10^2	2.10	0.40	0.22	31.38	0.87	0.06
2	5.61	1.15	588.9	2.9×10^2	2.47	0.040	0.50	74.42	2.07	0.13
3	5.80	0.29	538.9	1.7×10^2	2.22	0.035	0.31	41.85	1.16	0.08
4	5.40	0.65	684.3	4.8×10^3	3.68	0.054	7.03	1202	33.40	1.76
5	5.80	1.15	447.9	3.9×10^0	0.60	0.036	0.009	0.99	0.03	0.00
6	5.90	1.90	429.7	1.3×10^3	3.10	0.049	2.92	313.83	8.72	0.73
7	5.50	0.33	502.5	1.8×10^1	1.27	0.036	0.037	4.61	0.13	0.01
8	5.71	0.95	620.6	7.8×10^3	3.89	0.018	12.51	1942	53.96	3.13
9	5.80	2.46	647.5	1.7×10^4	4.22	0.016	25.83	4184	116.2	6.46
10	6.00	3.39	625.2	3.9×10^1	1.60	0.018	0.06	9.92	0.28	0.02
11	5.81	3.19	670.7	1.5×10^4	4.18	0.018	22.67	3802	105.6	5.67
12	5.70	1.94	716.1	7.8×10^3	3.89	0.019	3.11	558.07	11.63	0.78
13	5.50	1.69	784.3	7.8×10^2	2.89	0.024	1.60	313.83	6.54	0.40
14	5.60	1.94	697.9	7.1×10^2	2.85	0.017	1.34	235.34	4.90	0.34
15	5.85	1.61	725.2	3.9×10^2	2.60	0.021	0.41	74.42	1.55	0.10
16	5.72	1.21	697.9	4.8×10^3	3.68	0.021	3.20	558.07	11.63	0.80
17	5.71	2.12	697.9	8.5×10^3	3.93	0.021	0.69	120.23	2.50	0.17
18	5.92	1.91	611.5	8.5×10^2	2.93	0.019	0.10	16.03	0.33	0.03
19	5.31	1.65	647.5	1.8×10^4	4.27	0.018	23.47	380	79.21	5.87
20	5.15	1.91	584.3	3.9×10^3	3.60	0.010	5.60	819.14	17.07	1.40
21	5.17	2.15	584.3	4.3×10^3	3.64	0.017	5.60	819.14	17.07	1.40
22	5.10	1.99	570.6	1.6×10^3	3.22	0.007	3.55	507.02	10.56	0.89
23	5.55	3.29	638.8	2.0×10^3	3.31	0.015	3.17	507.02	10.56	0.79
24	5.5	3.15	666.1	2.0×10^3	3.31	0.016	1.88	313.83	6.54	0.47
25	5.4	0.65	629.8	2.9×10^3	3.47	0.015	3.54	558.07	11.63	0.89
26	5.5	2.45	647.9	5.2×10^3	3.72	0.015	5.05	819.14	17.07	1.26
27	5.05	3.29	629.8	2.0×10^3	3.31	0.015	1.99	313.83	6.54	0.50
28	5.2	3.64	652.5	8.5×10^3	3.93	0.024	13.11	2138	44.54	3.28
29	4.95	3.15	657.0	4.8×10^2	2.68	0.012	0.73	120.23	2.50	0.18
30	5.15	3.49	625.2	7.8×10^2	2.89	0.010	1.24	194.25	4.05	0.31

Table 3
Fitted Models for $\log_{10}$ AU by Setting Up Levels
for Factors x_1 = Sucrose, x_2 = Asparagine, x_3 = Phosphate, and x_4 = Tween-80 Under Extreme Conditions

$$\log_{10} \text{AU/mL} = 1.99 - 0.21x_2 + 1.80x_3 - 1.41x_4 + 0.23x_1x_2 + 0.36x_1x_3 + 1.47x_3x_4$$

	Factor Relation	Fitted Equation ($\log_{10}$ AU/mL)	Extreme Conditions		log AU/mL	Assays
Case 1:	$x_1 = +1; x_2 = +1$	$\text{AU/mL} = 2.01 + 2.16x_3 - 1.41x_4 + 1.47x_3x_4$	$x_3 = +1$	$x_4 = +1$	4.23	9
			$x_3 = -1$	$x_4 = -1$	2.73	10 and 15
			$x_3 = -1$	$x_4 = +1$	–3.03	
			$x_3 = +1$	$x_4 = -1$	4.10	11 and 19
Case 2:	$x_1 = -1; x_2 = -1$	$\log_{10} \text{AU/mL} = 2.43 + 1.44x_3 - 1.41x_4 + 1.47x_3x_4$	$x_3 = +1$	$x_4 = +1$	3.94	
			$x_3 = -1$	$x_4 = -1$	3.87	12
			$x_3 = -1$	$x_4 = +1$	–1.88	
			$x_3 = +1$	$x_4 = -1$	3.81	16
Case 3:	$x_1 = +1; x_2 = -1$	$\log_{10} \text{AU/mL} = 1.97 + 2.16x_3 - 1.41x_4 + 1.47x_3x_4$	$x_3 = +1$	$x_4 = +1$	4.19	
			$x_3 = -1$	$x_4 = -1$	2.69	13
			$x_3 = -1$	$x_4 = +1$	–3.07	
			$x_3 = +1$	$x_4 = -1$	4.06	17
Case 4:	$x_1 = -1; x_2 = +1$	$\log_{10} \text{AU/mL} = 1.55 + 1.44x_3 - 1.41\ x_4 + 1.47x_3x_4$	$x_3 = +1$	$x_4 = +1$	3.05	
			$x_3 = -1$	$x_4 = -1$	2.99	14
			$x_3 = -1$	$x_4 = +1$	0.17	
			$x_3 = +1$	$x_4 = -1$	2.93	18
Case 5:	$x_1 = 0; x_2 = 0$	$\log_{10} \text{AU/mL} = 1.99 + 1.80\ x_3 - 1.41x_4 + 1.47x_3x_4$				20
						21
			$x_3 = 0$	$x_4 = -1$	3.40	22
						23
						24

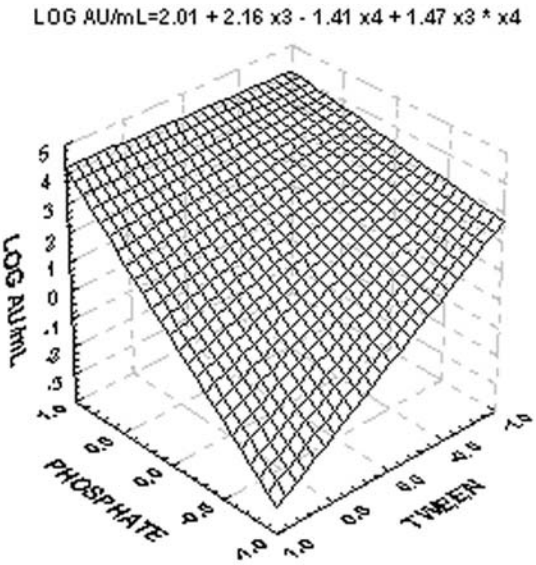

Fig. 1. Surface response representing effects of phosphate (x_3) and Tween-80 (x_4) on nisin production, keeping sucrose (x_1 = +1) and asparagine (x_2 = +1) at maximum levels, for fitted model $\log_{10}$ AU/mL = $2.01 + 2.16x_3 - 1.41x_4 + 1.47x_3x_4$.

positively and Tween-80 (x_4) negatively were similar in modulus, and were, respectively, 8.5 and 6.7 times greater than the negative individual influence of asparagine (x_2) in $\log_{10}$ AU/mL. The positive association of phosphate and Tween-80 ($1.47x_3x_4$) corresponded to 6.4 and 4.0 times, respectively, to the positive interactions between sucrose/asparagine ($0.23x_1x_2$) and sucrose/phosphate ($0.36x_1x_3$). The individual negative influence of Tween-80 (x_4) may be neutralized by the positive contribution of the phosphate (x_3), by keeping $x_4 = -1$ and $x_3 = +1$. The negative influence of asparagine (x_2) can also be neutralized by keeping $x_2 = +1$ and $x_1 = +1$, in the interaction of sucrose/asparagine ($0.23x_1x_2$). Furthermore, the nisin activity would depend on the sucrose level and on the strong interaction between phosphate and Tween-80. It was verified that the interactions between phosphate and Tween-80, sucrose and asparagine, and sucrose and phosphate contributed to the improvement in nisin activity, represented by the surface response in Fig. 1.

The best interval of proportions between nutrients (C = sucrose, N = asparagine, P = phosphate, T = Tween-80), which led to an improvement in the nisin production, was also verified (Table 1). These proportions were statistically evaluated by the polynomial model applied to the cases established (Table 3). In the condition of sucrose and asparagine kept at maximum levels (x_1 = +1 and x_2 = +1), case 1, nisin activity was expressed by the fitted model, equation 1:

$$\log_{10} \text{AU/mL} = 2.01 + 2.16x_3 - 1.41x_4 + 1.47x_3x_4. \quad (1)$$

For phosphate maintained at maximum level (x_3 = +1), independent of Tween-80 being at maximum (x_4 = +1) or minimum (x_4 = –1) level, the production of nisin reached its best activity between 4.10 $\log_{10}$ AU/mL and 4.23 $\log_{10}$ AU/mL. For these conditions, the proportions between the nutrients were: C/N = 0.17, C/P = 0.69, and N/P = 4.17, which were adopted as optimum for maximum specific nisin productivity (calculated for 2.5 mg of pure nisin preparation with a specific activity of 1.0×10^5 AU/mL) of about 6.0 mg/mg of DCW. Assays 9, 11 and 19 confirmed this with nisin titers, of ($\log_{10}$ AU/mL) 4.22, 4.18, and 4.27 (Table 2), respectively, although the ratio for T/P decreased from 0.37 to 0.06 (Table 1). For the minimum concentrations of phosphate and Tween-80, the ratios of C/P (2.08) and N/P (12.50) increased three times in relation to those adopted as optimum. This condition indicated phosphate as a limiting factor, reducing nisin activity around two log cycles (2.73 $\log_{10}$ AU/mL, Table 3, case 1), which is shown in assay 15, with nisin activity at 2.60 $\log_{10}$ AU/mL (Table 2). By maintaining phosphate (x_3 = –1) at the minimum concentration but Tween-80 at maximum concentration, the presence of nisin was kept at the basal concentration during the biomass growth, as occurred in assay 10 with a detection of 1.60 $\log_{10}$ AU/mL (Table 2), when the ratio T/P = 0.35 was twice that (T/P = 0.17) obtained for assay 15 (Table 1). Even the relationships between the other nutrients were the same for both assays 10 and 15. Lower T/P ratios showed a tendency to improve the nisin activity. Therefore, the original amount of Tween-80 presented in the MRS medium was sufficient to support a good nisin activity in relation to the phosphate concentration range (6–18 g/L) added.

For both sucrose (x_1 = –1) and asparagine (x_2 = –1) kept at the minimum levels, corresponding to case 2 (Table 3), the nisin activity fitted model (2) as follows was:

$$\log_{10} \text{AU/mL} = 2.43 + 1.44x_3 - 1.41x_4 + 1.47x_3x_4. \quad (2)$$

Equivalence was observed regarding nisin activity, by holding phosphate at minimum or maximum concentrations. However, Tween-80 should be kept at the minimum concentration when phosphate is also maintained at minimum concentration. In these situations, nisin activity ranged from 3.81 to 3.94 $\log_{10}$ AU/mL, confirmed in Table 2, for assays 12 (3.89 $\log_{10}$ AU/mL) and 16 (3.68 $\log_{10}$ AU/mL). In this case, the ratio C/N = 0.67 was constant and four times greater than that (C/N = 0.17) adopted as optimum, C/P = 0.28 was 2.5 times lower than the optimum ratio (C/P = 0.69), and N/P = 0.42 was 10 times lower than the one selected (N/P = 4.17), indicating that phosphate was excessive in relation to the other nutrients. The inhibition of nisin production will occur by keeping phosphate at the minimum level and Tween-80 at the maximum level, confirming the dependence of phosphate on Tween-80. For sucrose and asparagine held at minimum concentrations, the maximum specific nisin productivity decreased to about 0.80 mg/mg of DCW (assays 12 and 16, Table 2).

By establishing the concentrations of sucrose at x_1 = +1 and asparagine at x_2 = –1, case 3 (Table 3), nisin activity was estimated by the fitted model (3):

$$\log_{10} AU = 1.97 + 2.16x_3 - 1.41x_4 + 1.47x_3x_4 \quad (3)$$

For phosphate at $x_3 = +1$, independent of Tween-80 concentration ($x_4 = \pm 1$), the estimated nisin activity was equally maximum, ranging from 4.06 to 4.19 $\log_{10}$ AU/mL, with a detected average of 1.0×10^4 AU/mL in assay 17 (Table 2). The nutrient proportion C/P = 0.69 remained equal to that of case 1, while C/N = 1.67 was 10 times greater and N/P = 0.42 was 10 times lower than the ratios of case 1, adopted as optimum. For the conditions carried out, it was verified that the proportion C/P was the most important and determinant relationship of nisin-secreting cells during biomass growth. Moreover, sucrose, the carbon source, was proved to be indispensable for cell development, originating in lactic acid, which, in turn, is neutralized by phosphate, maintaining the medium at a favorable pH interval (5.0–6.0) for the development of biomass and consequent production of nisin. By holding phosphate and Tween-80 at minimum levels, the estimated nisin titer decreased to 2.69 $\log_{10}$ AU/mL, confirmed by assay 13 providing 2.89 $\log_{10}$ AU/mL, for proportions of C/P = 2.08 and N/P = 1.25; phosphate was insufficient to neutralize the excess sucrose, which was out of balance in relation to asparagine. For phosphate at $x_3 = -1$ and Tween-80 $x_4 = +1$, nisin activity will not be detected.

Maintaining sucrose at $x_1 = -1$ and asparagine at $x_2 = +1$, case 4 (Table 3), the maximum activity of nisin was estimated by the fitted model (4):

$$\log AU = 1.55 + 1.44x_3 - 1.41x_4 + 1.47x_3x_4 \quad (4)$$

Activity ranged from 2.93 to 3.05 $\log_{10}$ AU/mL, for phosphate at maximum concentration. The nutrient ratios were C/N = 0.067, 10 times below the optimum proportion adopted (case 1); C/P varied from 0.28 to 0.83 and N/P from 4.17 to 12.50, reinforcing excess asparagine in relation to sucrose and phosphate. For Tween-80 at $x_4 = +1$, although the ratios were maintained, nisin decreased from 2.92 $\log_{10}$ AU/mL ($x_3 = +1$) to a nondetectable ratio. Assays 14 and 18 (Table 2) represented this deficiency in sucrose, attaining 2.85 and 2.93 $\log_{10}$ AU/mL, respectively, and dropping maximum specific nisin productivity to about 0.03 mg/mg of DCW.

Analysis of these cases (Table 3) showed that the proportions between nutrients, which favorably contributed to the attainment of better values of nisin, averaged 4.0 $\log_{10}$ AU/mL and were (1) C/N = 0.17, C/P = 0.69, N/P = 0.42; and (2) C/N = 1.7, C/P = 0.69, N/P = 4.7. The C/P was constant and there was a 10-fold variation between the other ratios. For these conditions, high specific productivity over 22 AU/mg of DCW corresponding to an average of 6.0 mg of nisin/mg of DCW, reinforced assays 09, 11, and 19 (Table 2). For proportions of nutrients in the interval between the optimum values adopted, which were $0.17 < C/N < 1.7$ and $0.42 < N/P < 4.16$, maintaining a narrower range of $0.69 < C/P < 0.83$, the nisin titer ranged from 2.6 to 3.9 $\log_{10}$ AU/mL for half the productivity of 3.0 mg of nisin/mg of DCW, as shown in assays 25–30. The results showed the importance of the addition of nutrients to the medium and in proportions, that led to setting the best batch fermentation conditions at uncontrolled pH.

Nisin production clearly parallels that of biomass, thus showing primary metabolite kinetics *(7,14)*. For sucrose and asparagine at maximum concentrations (Table 1), assays 4, 9, 11, and 19, nisin activity was already detectable after 12 h of growth (AU/mL: 4.81, 1.98, 7.77, and 6.41, respectively), increasing about 2 $\log_{10}$AU after 24 h growth (AU/mL: 2.98×10^2, 3.61×10^2, 7.06×10^2, 1.14×10^2, respectively). At the end of the exponential phase at maximum specific rates of 0.054, 0.016 (36 h of growth, assays 4 and 9), and 0.018 h^{-1} (48 h of growth, assays 11 and 19), the highest titers (AU/mL) of 4.81×10^3, 1.67×10^4, 1.52×10^4, 1.84×10^4 were attained, respectively, equivalent to a biomass (mg of DCW/L) of 684.3, 647.9, 670.7, 648.0, respectively, although the arbitrary nisin productivity was 133.6 AU/(L·h) for assay 4 and three times greater for assays 9 (464.48 AU/[L·h]), 11 (422.46 AU/[L·h]), and 19 (383.86 AU/[L·h]) (Table 2).

The maximum specific nisin productivity (calculated for 2.5 mg of pure nisin preparation with a specific activity of 1.0×10^5 AU/mL) attained approx 1.76, 6.46, 5.67, and 5.87 mg of nisin/mg of DCW, respectively, for assays 4, 9, 11 and 19, while the conversion of sucrose in lactic acid was higher for assay 4 ($Y_{p/s}$ = 37.10%), decreasing to 21.23, 22.89, and 14.83% without interfering with the pH of the medium (Table 2), indicating that sucrose was in excess even though at a concentration of 5 g/L. The lower consumption of sucrose, with optimum nisin expression, was ensured by the ratio of C/N of 0.17 (Table 1). Therefore, the estimated concentration of asparagine of 29 g/L corresponding to 5 g/L of sucrose (assay 4) would attain an average titer of 4.0 $\log_{10}$ AU/mL. Furthermore, the relation of nutrients satisfied every condition established, indicating that sucrose at 5 g/L and asparagine at 29 g/L would be correlated to sucrose at 12.5 g/L and asparagine at 75 g/L, for C/N = 0.17. Thus, the relations of nutrients adopted (C/N = 0.17, C/P = 0.69, N/P = 4.17) as reference led to the optimum specific nisin productivity of 6.56, 5.67, and 5.87 (mg of nisin/mg of DCW) for assays 9,11, and 19.

There is strong evidence of positive correlation between maximum specific nisin production and biomass formation by *L. lactis*, which depends on the balance of the nutrients present in the growth medium.

Nomenclature

Parameters

AU/L = arbitrary units of nisin per liter of broth.
AU/mg of DWC = specific nisin titer.
AU/mL = arbitrary units of nisin per milliliter of broth.
mg of DCW/L = milligrams of dried cell weight per liter of broth.
mg of nisin/L = 25 mg of nisin equivalent to 10^5 AU of nisin/g of Nisaplin (Sigma). The AU/mL obtained was converted to milligram of nisin per liter of cultured broth as a result of this equivalence.
mg of nisin/L = pure nisin estimated for total growth period.

mg of nisin/(L·h) = medium productivity of pure nisin for total growth period.
mg of nisin/mg of DWC = maximum specific nisin activity.
$Y_{s/p}$ = conversion factor of sucrose in lactic acid.
μX = exponential growth rate (h^{-1}).

Annex 1

Main equation:

$$\log_{10} \text{AU/mL} = 1.99 - 0.21x_2 + 1.80x_3 - 1.41x_4 + 0.23x_1x_2 + 0.36x_1x_3 + 1.47x_3x_4.$$

Independent variables:

x_1 = sucrose; x_2 = asparagine; x_3 = potassium phosphate; x_4 = Tween-80.

Statistical Coefficients

$p <$ 0.05 significance level
$n =$ 16 observations
$p =$ 7 coefficients
$R^2 =$ 0.96 multiple determination for (n –1) = 15 df
$R^2 =$ 0.936 (df = p – 1 = 6)
SE = 0.18 standard error (df = p – 1 = 6)
Model Student's $t_{(0.05/2)} >$ (2.45
Model F_{ratio} (37.74) > $F_{critical}$ (0.95; p – 1 = 6; n – p = 9) = 3.37

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