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Production of Nisin by *Lactococcus lactis* in Media with Skimmed Milk

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Abstract

Nisin is a bacteriocin that inhibits the germination and growth of Gram-positive bacteria. With nisin expression related to growth conditions of *Lactococcus lactis* subsp. *lactis*, the effects of growth parameters, media components, and incubation time were studied to optimize expression. *L. lactis* ATCC 11454 was grown (100 rpm at 30ºC for 36 h) in both M17 and MRS standard broth media (pH 6.0–7.0) supplemented with sucrose (1.0–12.5 g/L), potassium phosphate (0.13 g/L), asparagine (0.5 g/L), and sucrose (0.24 g/L), and diluted 1:1 with liquid nonfat milk. Liquid nonfat milk, undiluted, was also used as another medium (9% total solids, pH 6.5). Nisin production was assayed by agar diffusion using *Lactobacillus sake* ATCC 15521 (30ºC for 24 h) as the sensitive test organism. The titers of nisin expressed and released in culture media were quantified and expressed in arbitrary units (AU/L of medium) and converted into known concentrations of "standard nisin" (Nisaplin®, g/L). The detection of nisin activity was <0.01 AU/L in M17 and MRS broths, and 7.5 AU/L in M17 with 0.14% sucrose or 0.13% other supplements, and the activity increased to 142.5 AU/L in M17 diluted with liquid nonfat milk (1:1). The 25% milk added to either 25% M17 or 25% MRS provided the highest levels of nisin assayed.

Index Entries: Nisin; *Lactococcus lactis*; growth conditions; skimmed milk; *Lactobacillus sake.*

Introduction

Lantibiotics are small peptides characterized by the presence of an unusual amino acid, lanthionine. Jung *(1)* defines lantibiotics as bacteriocins produced by lactic acid bacteria that are divided into two subgroups, type A and type B. Nisin, a type A bacteriocin, is a 34-residue monomeric penta-

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cyclic peptide (3.4 kDa). Initially isolated by Mattick and Hirsh *(2)*, nisin is produced during the exponential growth phase of the lactic acid bacteria, *Lactococcus lactis* subsp. *lactis (3,4).*

Like all bacteriocins, nisin has been used as a natural food preservative *(3–6)* and also in the preservation of pharmaceutical and dental products *(7,8).* However, recent research has verified the potential use of nisin for therapeutic purposes, particularly in the treatment of stomach ulcers and colon infections for patients with immune deficiencies *(9,10).* Nisin strongly inhibits the germination of spores and the growth of a broad range of Gram-positive bacteria *(5,6).* The growth of a variety of Gram-negative bacteria can also be inhibited by nisin if the outer membrane is first destabilized by EDTA *(11).*

Nisin was approved by the Food and Drug Administration in 1988 as Generally Recognized as Safe *(12)*, satisfying the demands for natural foods with less chemical additives. The most promising and the most important group of natural inhibitors are the bacteriocins, with a high economical added value. Commercial nisin is marketed as Nisaplin® and is used in processed foods such as various cheese products, desserts, sausages, bologna, pasteurized liquid egg, sauces, and other products. Nisin is a safe, natural antimicrobial and is quite suitable for use in heat-treated or low-pH food products *(13).*

Thomas et al. *(14)* investigated the efficacy of nisin in controlling the growth of *Bacillus* and *Clostridium* spores, which survive cooking and commonly grow in mashed potatoes. Wirjantoro et al. *(15)* investigated the use of nisin in combination with reduced heat treatment for milk (117ºC for 2 s). Wandling et al. *(16)* evaluated the potential for altering the survival of spores in thermally treated skimmed milk by supplementing the milk with various concentrations of nisin. Vessoni Penna et al. *(6)* verified the influence of nisin on the kinetics of growth from *B. cereus* spores germinated in milk formula. A notable property of nisin is that its solubility and stability increase drastically with the lowering of pH. Nisin is stable at pH 2.0 and, at this pH, can be autoclaved at 121ºC for 15 min without inactivation *(17).*

The production of nisin is growth associated because expression occurs during the midexponential phase and increases until reaching a maximum level at the end of the exponential phase or at the beginning of the early stationary phase *(18–22).* Previous studies have shown that nisin is expressed continuously from the initial stages of growth to the stationary phase *(3,4).*

There is an extensive background of earlier work done to optimize nisin expression in *Lactococcus lactis,* and many of these studies, have described MRS and Ml7 broth for optimal cellular growth and expression *(18,19,23–25).*

The release of bacteriocin from the cells into the growth medium is dependent on the pH *(19,20).* At pH <6.0, more than 80% of the nisin

produced is released into the medium; at pH >6.0, most of the nisin is associated with the cellular membrane, but not the cytoplasm *(25).*

As a culture medium, the high nutritive content of cow's milk provides for excellent growth of *L. lactis* and for the cell's release of nisin into the medium. The cells ferment lactose to lactic acid, which causes a decrease in the medium pH, enhancing the release of nisin from the cells into the medium.

Cheigh et al. *(23)* studied growth parameters, such as the medium components in Ml7 broth, growth temperature, pH, and carbon source concentrations that affected bacteriocin production by *L. lactis* subsp. *lactis* A164. Chandrapatti and O'Sullivan *(25)* developed a rapid plate assay that enabled the quantitative assessment of growth parameters in M17-based media that influence nisin production by growing *L. lactis* directly on the solid medium containing the test factor. This assay was used to assess the influence of carbohydrates and salts on nisin production by *L. lactis* on a per cell basis, and the data were subsequently substantiated with broth cultures. Biwas et al. *(17),* Daba et al. *(18),* ten Brink et al. *(22),* MacGroarty and Reid *(26),* Toba et al. *(27)* related that MRS is a better medium for cell growth and bacteriocin expression. Different concentrations of nutritional supplements such as sucrose, lactose, and glucose could be related to nisin expression and release into the culture media by *L. lactis (26,27).* High concentrations of sucrose correlated to an increase in nisin expression at the end of the stationary phase *(4).*

Vessoni Penna and Moraes *(28)* studied the influence of sucrose with asparagine (7.5–75 g/L), potassium phosphate (6–18 g/L), and Tween-80 (1–6 g/L) added to MRS broth on nisin production by *L. lactis.* The formulations that improved nisin expression by *L. lactis* indicated that 5 g/L of sucrose with 29 g/L of asparagine in MRS was equivalent to the expression derived with a composition of 12.5 g/L of sucrose with 75 g /L of asparagine in MRS.

In the present study, we used 0.14% (1.4 g/L) and 12.5% (125 g/L) sucrose to determine whether these concentrations would result in the same extent of nisin expression. We also examined the use of sucrose and asparagine to determine the benefits of these supplements used simultaneously in media.

The utilization of synthetic MRS medium with or without sucrose, asparagine, and potassium phosphate showed that the expression of nisin is related to nutritional and growth factors, and that cellular growth and nisin expression are concomitant, from fermentation time to the beginning of stationary phase, about 36 h *(28),* the incubation used throughout this work.

With nisin expression related to growth conditions of *L. lactis* subsp. *lactis* ATCC 11454, the effects of culturing parameters, such as media components, incubation time, number of culture transfers to fresh medium, were evaluated to optimize the expression of nisin and release into media.

Materials and Methods

Strains and media

The nisin-producing strain of *L. lactis*. ATCC 11454 and the nisin-sensitive indicator strain of *Lactobacillis sake* ATCC 9221 were used. The cultures were maintained at –80ºC in MRS broth (Man Rugosa Shepeer-Bacto Lactobacilli MRS broth; Difco, Detroit, MI) with 40% (v/v) glycerol.

The experiments were performed utilizing the following media, as outlined in Tables 1 and 2:

1. Synthetic MRS broth (Difco) and Ml7 broth (Oxoid) at their standard concentrations per the manufacturer's recommendations.
2. MRS or Ml7 at half their standard concentrations (item 1) with 0.13% of supplements: 0.035% sucrose, 0.018% potassium phosphate, and 0.075% asparagine.
3. Ml 7 at half the standard concentration (item l) supplemented with 0.14% sucrose.
4. Ml7 at the standard concentration supplemented with 12.5% sucrose.
5. Skimmed milk (9.09% dry matter [DM], nonfat milk UHT; Premium, Parmalat,SP, Brazil).
6. MRS or Ml7 at 25% of their standard concentrations plus skimmed milk (25% MRS + 25% milk and 25% Ml 7 + 25% milk).
7. MRS or Ml7 at 17.36% of their standard concentrations plus 17.36% of skimmed milk and with 0.13% of supplements: 0.035% sucrose, 0.018% potassium phosphate, and 0.075% asparagine.
8. MRS or Ml7 at 17.86% of their standard concentrations plus 17.86% of skimmed milk and supplemented with 0.14% sucrose.

Prior to use in defined experimental media, 100 µL of frozen glycerol *L. lactis* stock cultures was grown in either MRS or Ml 7 ("preinoculum"; 50 mL of broth in 250-mL flasks) and incubated in a rotary shaker (100 rpm) at 30ºC for 36 h. From the preinoculum, 5-mL aliquots of the cell suspension were transferred into 50 mL of each experimental medium in 250 mL flasks and incubated for another 36 h (100 rpm of 30ºC). Cultures were transferred five times (l00 rpm at 30ºC for 36 h) using 5-mL aliquots of broth culture for each new volume of the respective medium, as shown in Tables 3 and 4.

After every 36-h incubation period, 30 mL of cell suspensions was aseptically withdrawn from the flasks and tested for pH, cellular density, colony number, and nisin concentrations. Every type of media examined was performed in triplicate.

Measurement of pH

The pH was measured in 10 mL of suspension with an Accumet AR20 pH/mV/conductivity meter (Fisher, Fairview, NJ) calibrated with standard buffer solutions (Merck; pH 4.0, 7.0, 10.0) at 25ºC.

Table 1
Composition of MRS Broth, Skimmed Milk, and Supplements

Composition g/l00 mL	MRS basic composition	MRS with supplements	Milk	MRS with milk	MRS with milk + supplements
MRS broth					
Yeast extract	0.50	0.35	—	0.13	0.09
Magnesium sulfate	0.01	4.8E-03	—	2.5E-03	1.7E-03
Proteose peptone	1.00	0.48	—	0.25	0.17
Dextrose	2.00	0.96	—	0.50	0.35
Meat extract	1.00	0.48	—	0.25	0.17
Ammonium citrate	0.20	0.10	—	0.05	0.03
Potassium phosphate	0.20	0.10	—	0.05	0.03
Tween-80	0.10	0.05	—	0.03	0.02
Manganese sulfate	0.01	4.8E-03	—	2.5E-03	1.7E-03
Sodium acetate	0.50	0.24	—	0.13	0.09
Supplements					
Sucrose	—	0.03	—	—	0.03
Potassium phosphate	—	0.02	—	—	0.02
Asparagine	—	0.08	—	—	0.08
Skimmed milk					
Carbohydrates	—	—	5.00	1.25	0.87
Protein	—	—	3.00	0.75	0.52
Iron	—	—	0.45	0.11	0.08
Calcium	—	—	0.20	0.05	0.03
Cholesterol	—	—	0.25	0.06	0.04
Total fats	—	—	0.01	2.5E-03	1.7E-03
Saturated fats	—	—	0.12	0.03	0.02
Sodium	—	—	0.05	0.01	8.7E-03
Vitamin A	—	—	0.01	1.5E-03	1.0E-03
Vitamin D	—	—	3.8E-04	9.4E-05	6.5E-05
Total solids (g/100mL)	5.52	2.89	9.09	3.65	2.64

Table 2
Composition of Ml 7 broth, skimmed milk and supplements

Composition g/100 mL	M17 basic composition	M17 with supplements	M17 with sucrose	M17 with sucrose	Milk	M17 with milk	M17 with milk + supplements	M17 with milk + sucrose
M17 broth								
Tryptone	0.53	0.37	0.38	0.53	—	0.13	0.09	0.10
Yeast extract	0.26	0.18	0.19	0.26	—	0.07	0.05	0.05
Magnesium sulfate	0.03	0.02	0.02	0.03	—	0.01	4.5E-03	4.6E-03
Soy protein	0.53	0.37	0.38	0.53	—	0.13	0.09	0.09
Meat digest	0.53	0.37	0.38	0.53	—	0.13	0.09	0.09
Ascorbic acid	0.05	0.04	0.04	0.05	—	0.01	0.01	0.01
Disodium glycerophosphate	2.00	1.39	1.43	2.00	—	0.50	5	0.36
Supplements								
Sucrose	—	0.03	0.14	12.50	—	—	0.03	0.14
Potassium phosphate	—	0.02	—	—	—	—	0.02	—
Asparagine	—	0.08	—	—	—	—	0.08	—
Skimmed Milk								
Carbohydrates	—		—	—	5.00	1.25	0.87	0.89
Protein	—	—	—	—	3.00	0.75	0.52	0.54
Iron	—	—	—	—	0.45	0.11	0.08	0.08
Calcium	—	—	—	—	0.20	0.05	0.03	0.04
Cholesterol	—	—	—	—	0.25	0.06	0.04	0.04
Total fats	—	—	—	—	0.01	2.5E-03	1.7E-03	1.8E-03
Saturated fats	—	—	—	—	0.12	0.03	0.02	0.02
Sodium	—	—	—	—	0.05	0.01	0.01	0.01
Vitamin A	—	—	—	—	0.01	1.5E-03	1.0E-03	1.1E-03
Vitamin D	—	—	—	—	3.8E-04	9.4E-05	6.5E-05	6.7E-05
Total solids g/100 mL	3.93	2.87	2.96	16.43	9.09	3.24	2.38	2.46

Table 3
Activity, Productivity, and Specific Production of Nisin for Every Transfer After 36 h of Growth of *L. lactis* ATCC 11454 Transferred to a New Medium with Milk Added

Medium Composition	Preinoculum	pH	Transfer	Activity[a] (AU/L)	Nisin[b] (g/L)	Specific production (mg DCW/mg) × 10^{-3}	Productivitv [mg·h][c] (mg DCW/[g·h]) ×10^{-3}
100% Milk 1	MRS	5.1	1	4.1	0.1	2.8	7.6
(pH 6.80)		4.2	2	16.3	0.4	11.3	26.2
		4.3	3	35.4	0.9	24.6	65.7
		4.7	4	22.2	0.6	15.4	39.8
		4.8	5	16.3	0.4	11.3	30.6
100% Milk 2	M17	5.5	1	26.1	<0.01	<0.01	0.1
(pH 6.82)		4.8	2	4.1	0.1	2.8	11.4
		4.9	3	7.5	0.2	5.2	16.3
		4.9	4	26.0	0.6	18.0	48.6
		4.7	5	19.1	0.5	13.2	26.9
25% M17 + 25% milk	M17	6.2	1	16.3	0.4	11.3	30.6
(pH 6.17)		4.8	2	65.7	1.6	45.6	105.4
		4.7	3	65.7	1.6	45.6	122.1
		5.0	4	41.3	1.0	28.7	73.9
		4.8	5	142.5	3.6	99.0	266.8
25%MRS + 25%milk	MRS	4.7	1	35.4	0.9	24.6	66.4
(pH 6.12)		4.6	2	142.5	3.6	99.0	400.7
		4.7	3	35.4	0.9	24.6	76.8
		4.7	4	89.6	2.2	62.2	167.7
		4.8	5	35.4	0.9	24.6	50.1

(Continued…)

Table 3 *(Continued)*

Medium Composition	Preinoculum	pH	Transfer	Activity[a] (AU/L)	Nisin[b] (g/L)	Specific production (mg DCW/mg) × 10^{-3}	Productivitv [mg·h][c] (mg DCW/[g·h]) × 10^{-3}
17.36% MRS + 17.36% milk	MRS	4.5	1	<0.01	<0.01	<0.01	<0.01
+ 0.13% Supplements		6.0	2	<0.01	<0.01	<0.01	<0.01
(pH 6.35)		5.4	3	<0.01	<0.01	<0.01	<0.01
		6.3	4	6.4	0.2	4.5	11.5
		5.4	5	6.4	0.2	4.5	12.1
17.36%M17 + 17.36% milk	M17	6.5	1	<0.01	<0.01	<0.01	<0.01
+ 0.13% Supplements		5.1	2	<0.01	<0.01	<0.01	<0.01
(pH 6.76)		5.1	3	<0.01	<0.01	<0.01	<0.01
		57	4	0.9	<0.01	0.6	1.5
		5.62	5	2.5	0.1	1.8	4.8
17.86% M17 + 17.86% milk	M17	5.7	1	<0.01	<0.01	<0.01	<0.01
+ 0.14% sucrose		5.1	2	<0.01	<0.01	<0.01	<0.01
(pH 6.74)		5.1	3	<0.01	<0.01	<0.01	<0.01
		5.3	4	8.8	0.2	6.1	15.7
		5.3	5	7.5	0.2	5.2	14.1

[a] Arbitrary unity per liter: $AU/L = 10^{(0.2689 \times H + 1.3893)}$, in which H= diameter of the halo (mm), and the SD = 0.4–0.5.

[b]Nisin concentration: gL = (*x*) *0.025/1000, in which *x* Activity (AU/mL) and 0.025 = the conversion factor of the standard nisin solution (0.025 mg/mL = 10^3 AUmL).

[c] Specific production = (*x*)/(DCW), in which *x* = nisin concentration (mgL), and DCW = dry cell weight (mgL) = (mg nisin. DCWmg) × 10^{-3}. Productivity: (mg/mg DCW·h), 10^{-3}= (*x*)/36, in which *x* = specific production for 36 h of incubation.

Table 4
Activity, Productivity, and Specific Production of Nisin for Every Transfer
After 36 h of Growth of *L. lactis* ATCC 11454 Transferred to a New Medium With Synthetic Media.

Medium Composition	Preinoculum	pH	Transfer	Activity[a] (AU/L)	Nisin[b] (gL)	Specific production[c] (mg DCW/mg) × 10^{-3}	Productivity[d] (mg DCW/[mg·h]) × 10^{-3}
100% MRS (pH 6.5)	MRS	6.1	1	<0.01	<0.01	0.6	<0.01
		6.2	2	<0.01	<0.01	0.4	<0.01
		5.7	3	<0.01	<0.01	0.4	<0.01
		5.3	4	<0.01	<0.01	0.5	<0.01
		5.1	5	<0.01	<0.01	2.5	<0.01
100% M17 (pH 6.9)	M17	7.1	1	<0.01	<0.01	<0.01	<0.01
		7.3	2	<0.01	<0.01	< 0.01	<0.01
		6.8	3	<0.01	<0.01	<0.01	<0.01
		7.0	4	<0.01	<0.01	<0.01	<0.01
		7.2	5	<0.01	<0.01	<0.01	<0.01
50% MRS + 0.13% supplements (pH 6.13)	MRS	5.9	1	<0.01	<0.01	<0.01	<0.01
		4.7	2	<0.01	<0.01	<0.01	<0.01
		4.9	3	<0.01	<0.01	<0.01	< 0.01
		4.7	4	1.5	<0.01	2.7	1.1
		4.8	5	9.2	0.2	17.3	6.4
50% M17 + 0.13% supplements (pH 6.28)	M17	6.0	1	<0.01	<0.01	<0.01	<0.01
		6.0	2	<0.01	<0.01	<0.01	<0.01
		6.1	3	<0.01	<0.01	<0.01	<0.01
		6.1	4	7.5	0.2	13.4	5.2
		6.3	5	<0.01	<0.01	<0.01	<0.01

(Continued…)

Table 4 *(Continued)*

Medium Composition	Preinoculum	pH	Transfer	Activity[a] (AU/L)	Nisin[b] (gL)	Specific production[c] (mgDCW/mg) × 10^{-3}	Productivity[d] (mg DCW/[mg·h]) × 10^{-3}
50% M17 + 0.14%	M17	5.5	1	0.6	<0.01	1.2	0.4
sucrose (pH 6.94)		5.6	2	1.1	<0.01	1.8	0.8
		5.7	3	0.8	<0.01	1.5	0.6
		5.9	4	7.5	0.2	13.4	5.2
		6.0	5	6.0	0.2	11.3	4.2
100% M17 + 12.5%	M17	4.5	1	<0.01	<0.01	<0.01	<0.01
sucrose (pH 6.27)		4.5	2	<0.01	<0.01	<0.01	<0.01
		4.5	3	0.2	<0.01	0.4	0.2
		4.5	4	0.2	<0.01	0.4	0.2
		4.4	5	11.4	0.3	21.4	7.9

[a] Arbitrary units per liter: AU/L = $10^{(0.2689 \times H + 1.3893)}$, in which H = diameter of halo (mm), and the SD = 0.4–0.5.
[b]Nisin concentration: g/L = $(x) \times 0.025/1000$, in which x = activity (AU/mL) and 0.025 = the conversion factor of the standard nisin solution (0.025 mg/mL = 10^3 AU/mL).
[c]Specific production = $(x)/(DCW)$, in which x = nisin concentration (mg/L), and DCW = dry cell weight (g/L)=(mg nisin/mg DCW) × 10^{-3}.
[d]Productivity: (mg/mg DCW·h) × 10^{-3}= $(x)/36$, in which x = specific production for 36 h of incubation.

Measurement of Biomass Concentration

The cellular biomass concentration, expressed in milligrams of dried cell weight (DCW) per liter of broth, was determined from the optical density (OD) at 660 nm (OD_{660}) in a 1-cm path-length quartz cuvet in a spectrophotometer (Beckman DU-600). The OD_{660} readings were calibrated against a standard dried cellular concentration curve of *L. lactis*, which was obtained by the gravimetric method of the biomass (mg/L) held on the surface of a 0.22-μm membrane (Millipore). The equation for the calibration curve ($R^2 = 0.998$) was as follow: $OD_{660} = 0.0145 + 0.0022 \times DCW$ or DCW (mg/L) = $[(OD_{660}) + 0.0145)]/(0.0022)$.

Measurement of Colony Number

Culture populations were assayed by the plate count method, expressed in colony-forming units (CFU) per milliliter of broth in Plate Count Agar (Difco) at 30ºC for 24 h. Cell numbers were related to the reference curve associating OD_{660} to DCW (mg DCWL) of the same suspension, in which $OD_{660} = 0.01$ was equivalent to 10^4 CFU/mL.

Detection of Nisin Activity

For detection of nisin activity, the cell suspension was centrifuged at 12,000*g* for 10 min at 25ºC, and the supernatant collected was filtered through a 0.22 = μm membrane filter (Millipore). The titers of nisin expressed and released in culture media were quantified and expressed in arbitrary units (AU/L of medium) by the agar diffusion assay *(6,28)* utilizing *L. sake* as a sensitive indicator microorganism. *L. sake* was grown in MRS broth and incubated (l00 rpm at 30ºC for 24 h). A 1.5-mL aliquot of the suspension (OD_{660} = 0.7) was transferred and mixed with 250 mL of soft agar (MRS broth with 0.8% of bacteriologic-grade agar). Each 20 mL of inoculated medium was transferred to Petri plates (100 mm). After the agar solidified, 3-mm wells were cut out with a sterile metal pipe with a 5-mm total diameter.

From every medium, 50 = μL of culture supernatant from centrifuged *L. lactis* suspension was transferred into the wells on the surface of the *L. sake* inoculated agar. For the milk-supplemented media, culture supernatant, or with without a prior adjustment to pH 2.5 with 0.2 *N* HC1, was transferred into the wells. The adjustment to pH 2.5 prior to centrifugation was done to coagulate the milk, releasing nisin into a clear supernatant (after centrifugation) to facilitate detection of nisin. This same pH adjustment was done for solutions used for the standard nisin curve. *L. sake* growth was not inhibited by nisin-free HC1 solution (pH 2.5) added to the wells, confirming earlier studies *(6,28).*

The plates, without inversion, were incubated at 30ºC for 24 hs. After this period, inhibition halos, zones without *L. sake* growth, were measured in four directions and the average diameters (±0.5 mm) of the halos related to the arbitrary activities (AU/L) of nisin formed by the respective cultures,

as determined from standard curves using purified nisin as the reference. The relation between arbitrary units (AU/L) and international units (IU/L) was determined by using Nisaplin (a commercial purified nisin preparation containing 25 mg of nisin/g of Nisaplin , corresponding to 10^6 lU/g of Nisaplin Aplin & Barret Ltda, Beaminster, UK, distributed by Sigma). A standard solution of nisin was prepared by dissolving 1 g of Nisaplin® in 10 mL of 0.02 *N* HCl with 0.75% (w/v) NaCl (pH 1.6–1.8). The solution was autoclaved at 121°C for 15 min and stored at 4°C. Further dilutions of the standard were made as necessary by diluting in 0.02 *N* HCl. With the standard curve, the concentrations of standard nisin (10^0–10^5 AU/L) were related by the diameter of the inhibition halo (*H*, mm), and the activity of nisin from cells grown in the experimental media was determined and expressed in arbitrary units per liter (10^o–10^5AU/L). Based on the calibration curves between AU/L and IU/L, 1.09 ± 0.17 AU corresponded to 1.0 IU (40 IU = 1 μg of pure nisin A).

For every medium studied, the arbitrary units were calculated from equations, as follows:

1. For the MRS and M17 broths, the relation was AU/L = $10^{(0.01847 \times H + 0.8259)}$.
2. For media containing milk, without adjusting the pH of the culture supernatant, the relation between AU/L and inhibition halo (*H*, mm) was AU/L = $10^{(0.2689 \times H + 1.3893)}$.
3. For media with the pH of the supernatant adjusted to 2.5, the relation between AU/L and inhibition halo (H, mm) was AU/L = $10^{(0.478 \times H + 0.7388)}$.

Using the standard solutions for calibration of nisin activity in all the assays, 0.025 mg of nisin corresponded to 10^3 IU/ml. The activity of nisin expressed in AU/L was converted for nisin in grams per liters through the relation *(3)*

$$\text{Nisin}\,(\text{g/L}) = \frac{(z \times 0.025)}{1000}$$

In which *z* = AU/L. The concentration of nisin was expressed in grams per liters, and in the production of nisin (g/[L·h]) related the formation of nisin in grams per liter to a 36-h incubation period. The amount of nisin related to cellular mass (DCW) gave specific production and productivity. The specific production (mg/mg) is the ratio of nisin concentration (mg/L) and the cellular mass (mg DCW/L). Productivity was expressed in milligrams of nisin per milligram of DCW times hour as the ratio of the hourly milligrams of nisin (mg/[L·h]) and cellular mass (mg DCW*)*, for 36 h of incubation.

Results and Discussion

With the objective of studying the influence of medium components on nisin expression, a strain of *L. lactis* was transferred five consecutive

times after 36-h intervals of incubation with the same culture medium and incubated under the same conditions. The media additives and incubation times were derived from prior studies carried out in our laboratory. The activity, productivity and specific production of nisin for media, with or without milk, are presented in Tables 3–6. Tables 5 and 6 show activity, productivity and specific production of nisin from every transfer after incubating at 100 rpm and 30ºC for 36 h when *L. lactis* cultures were transferred to fresh media: the collected supernatant was adjusted to pH 2.5 prior to detection of nisin. This pH adjustment was done to coagulate the milk, releasing nisin into a clear supernatant (after centrifugation) to facilitate detection of nisin. This same pH adjustment was done for solutions used for the standard nisin curve. *L. sake* growth was not inhibited by nisin-free HC1 solution (pH 2.5) added to the wells.

Cheigh et al. *(23)* observed the highest nisin activity early in the stationary phase (20 h, 30ºC) of *L. lactis* during batch fermentation in M17 broth (pH 6.0) with 3% lactose added. In fact, M17 broth with 3% lactose resulted in an eight-fold greater nisin expression than either M17 supplemented with 0.5% glucose or in MRS broth. These investigation confirmed low levels of nisin expression in both MRS and M17 broth, although these media favored cellular growth, with similar results obtained in the present study (10^7 – 10^9 CFU/mL). Chandrapatti and O'Sullivan *(25)* observed a 50% increment in nisin expression using sucrose as the carbon source in M17 broth for *L. lactis* culturing, over two transfers. They observed that glucose was the optimal carbon source tested, with glycerol the least suitable. They also verified that the incorporation of either sodium or potassium phosphate into a synthetic medium did not improve nisin production and release into the media.

The results of our study obtained in all assays demonstrated that supplements, principally sucrose used with standard concentrations of Ml7 and MRS (Tables 4 and 6), were essential for the expression and release of nisin by *L. lactis* into the culture media. Sucrose at a concentration of 0.14% in M17 (at 50% of the standard concentration; Table 4) directly enhanced the expression of nisin and was shown to be the minimum concentration necessary to improve nisin expression and release into media for all five transfers. Therefore, nisin expression and release into the media was greater with 0.14% sucrose added to Ml7 (50%) than with 12.5% sucrose added (standard concentration; Table 4), in which nisin was barely detectable at the first and second transfers, low for the third and fourth transfers (< 0.1 g/L) then increasing 25-fold (0.3 g/L) in the fifth transfer.

The culture media with milk provided better conditions for *L. lactis* growth and its concomitant expression of nisin, in relation to MRS and M17 with or without 0.013% supplements or 0.14% sucrose (Tables 3 and 4).

Milk alone (9.09% DM) favored nisin expression and release into the media for all five transfers, from 0.4 to 0.9 g/L , similar to that attained at

Table 5
Activity, Productivity, and Specific Production of Nisin for Every Transfer
After 36 h of Growth of *L. lactis* ATCC 11454 Transferred to a New Medium with Milk Added and pH Adjusted to 2.5

Medium Composition	Preinoculum	Transfer	Activity[a] (AU/L)	Nisin[b] (g/L)	Specific production[c] (mg DCW/mg) × 10^{-3}	Productivity[d] (mg DCW/[mg·h]) × 10^{-3}
100% Milk 1 (pH 6.80)	MRS	1	1.2	<0.01	83.7	2.3
		2	2.9	0.1	168.3	4.7
		3	12.1	0.3	811.3	22.5
		4	12.1	0.3	782.0	21.7
		5	2.2	0.1	147.7	4.1
100% Milk 2 (pH 6.82)	M17	1	1.4	<0.01	205.2	5.7
		2	0.8	<0.01	81.7	2.3
		3	2.2	0.1	171.2	4.8
		4	9.1	0.2	614.8	17.1
		5	3.9	0.1	197.4	<0.01
25% M17 + 25% milk (pH 6.17)	M17	1	1.6	<0.01	111.3	3.1
		2	16.1	0.4	932.1	25.9
		3	5.2	0.1	344.7	9.6
		4	18.6	0.5	1199.7	33.3
		5	3.4	0.1	226.5	6.3
25% MRS + 25% milk (pH 6.12)	MRS	1	2.2	0.1	148.1	4.1
		2	6.9	0.2	396.1	11.0
		3	7.9	0.2	528.8	14.7
		4	10.5	0.3	678.0	18.8
		5	50.5	1.3	3405.4	94.6

17.36% MRS + 17.36% milk + 0.13%supplements (pH 6.35)	MRS	1	<0.01	<0.01	0.4	<0.01
		2	<0.01	<0.01	0.3	<0.01
		3	<0.01	<0.01	0.4	<0.01
		4	2.2	0.1	141.2	3.9
		5	2.2	0.1	147.7	4.1
17.36% M17 + 17.36% milk + 0.13%supplements (pH 6.76)	M17	1	<0.01	<0.01	0.4	<0.01
		2	<0.01	<0.01	0.3	<0.01
		3	<0.01	<0.01	0.4	<0.01
		4	2.5	0.1	162.8	4.5
		5	28.6	0.7	1924.7	53.5
17.86% M17 + 17.86% milk + 0.14% sucrose (pH 6.74)	M17	1	<0.01	<0.01	0.4	<0.01
		2	<0.01	<0.01	0.3	<0.01
		3	<0.01	<0.01	0.4	<0.01
		4	12.1	0.3	782.0	21.7
		5	2.5	0.1	170.3	4.7

[a]Arbitrary units per liter: $AU/L = 10^{(0.2478 \times H + 0.7388)}$, in which H = diameter of the halo (mm) and the SD = 0.4–0.5.

[b] Nisin concentration: $g/L = (x) \times 0.025/1000$, in which x = activity (AU/mL) and 0.025 = the conversion factor of the standard nisin solution ($0.025\ mg/mL = 10^{-3}\ AU/mL$).

[c]Specific production = $(x)/(DCW)$, in which x = nisin concentration (mg/L) and DCW = dry cell weight (mg/L) = (mg nisin DCW/mg) $\times 10^{-3}$.

[d]Productivity: (mg/mg DCW·h) $\times 10^{-3} = (x)/36$, in which x = Specific production for 36 h of incubation.

Table 6
Activity, Productivity, and Specific Production of Nisin for Every Transfer After 36 h of Growth of *L. lactis* ATCC 11454 Transferred to a New Medium with Synthetic Media and pH Adjusted to 2.5

Medium Composition	Preinoculum	Transfer	Activity[a] (AU/L)	Nisin[b] (g/L)	Specific production[c] (mg DCW/mg) × 10^3	Productivity[d] (mg DCW/[mg·h]) × 10^{-3}
MRS (pH 6.5)	MRS	1	<0.01	<0.01	1.0	<0.01
		2	<0.01	<0.01	0.7	<0.01
		3	0.1	<0.01	6.7	0.2
		4	0.2	<0.01	13.6	0.4
		5	0.5	<0.01	26.7	<0.01
100% M17 (pH 6.9)	M17	1	<0.01	<0.01	0.5	<0.01
		2	<0.01	<0.01	0.4	<0.01
		3	<0.01	<0.01	0.5	<0.01
		4	<0.01	<0.01	0.5	<0.01
		5	<0.01	<0.01	0.5	<0.01
50% MRS + 0.13% supplements (pH 6.13)	MRS	1	<0.01	<0.01	0.5	<0.01
		2	<0.01	<0.01	0.4	<0.01
		3	0.3	<0.01	18.5	0.5
		4	0.5	<0.01	30.3	0.8
		5	5.4	0.1	366.0	10.2

50% M17 + 0.13% supplements (pH 6.28)	M17	1	<0.01	<0.01	0.5	<0.01
		2	<0.01	<0.01	0.4	<0.01
		3	<0.01	<0.01	0.5	<0.01
		4	2.9	0.1	184.9	5.1
		5	0.8	<0.01	54.0	1.5
50% M17 + 0.14% sucrose (pH 6.94)	M17	1	0.01	<0.01	0.5	<0.01
		2	1.1	<0.01	63.6	1.8
		3	<0.01	<0.01	0.5	<0.01
		4	4.9	0.1	314.7	8.7
		5	1.4	<0.01	91.9	2.6
100% M17 + 12.5% sucrose (pH 6.27)	M17	1	<0.01	<0.01	0.5	<0.01
		2	<0.01	<0.01	0.4	<0.01
		3	1.9	<0.01	125.4	3.5
		4	1.9	<0.01	120.9	3.4
		5	6.7	0.2	452.7	12.6

[a]Arbitrary units per liter: AU/L = $10^{(0.2478 \times H + 0.7388)}$, in which H = diameter of the halo (mm), and the SD = 0.4–0.5.

[b]Nisin concentration: g/L - (x) × 0.025/1000, in which x = activity (AU/mL) and 0.025 = conversion factor of the standard nisin solution (0.025 mg/mL = 10^{-3} AU/mL).

[c]Specific production = (x)/(DCW). In which x = nisin concentration (mg/L) and DCW = dry cell weight (mg/L) = (mg nisin DCW/mg) × 10^{-3}.

[d]Productivity: (mg/mg DCW·h) × 10^{-3} (x)/36, in which x = specific production for 36 h of incubation.

the first transfer for both 25% MRS + 25% milk and 25% Ml7 + 25% milk, with the latter medium providing the highest nisin concentration, 3.6 g/L after the fifth transfer. A dilution of these media to a 17.36% concentration provided levels of nisin activity (0.1 g/L for 17.36% M17 + 17.36% milk and 0.2 g/L for 17.36% MRS + 17.36% milk) five times lower than those detected relative to 25% concentration for the media assayed (Table 3).

The preinoculum of *L. lactis* in MRS (media used prior to the first transfer) stimulated the expression of nisin in "milk 1" media at the first transfer. Using M17 as the preinoculum medium for "milk 2" cultures, nisin was undetectable at the first transfer, although both "milk 1" and "milk 2" favored nisin expression and release during the fourth and fifth transfers, with nisin concentrations very similar for both milk media.

The successive transfer of cells into fresh media may provide conditions for greater nisin production above the "ceiling concentration" as noted by Kim et al. *(29)*; the repeated dilutions may eliminate the inhibiting effect of high nisin concentrations on the producing organism.

Adjusting the culture supernatant to a final pH of 2.5 did not improve the detection of nisin throughout the assays; this pH adjustment decreased the activity of nisin (Tables 5 and 6). In many assays (Table 4) even with no adjustment of pH that was lower than 6.0, the detection of nisin activity was not significant ($p < 0.05$) considering MRS and Ml7 at standard concentration.

The values obtained with standard nisin solutions adjusted to pH 2.5 were lower than the standard nisin without this adjustment (calibration curves); the values obtained for nisin in the samples with pH adjusted to 2.5 were lower than the results obtained from samples without the pH adjustment.

These results demonstrate that, nisin production is not directly related to cell number because lower cell populations provided higher nisin concentrations. The 25% milk added to either 25% Ml7 or 25% MRS provided the highest levels of nisin assayed.

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