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Detection of Nisin Expression by *Lactococcus lactis* Using Two Susceptible Bacteria to Associate the Effects of Nisin With EDTA

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Abstract

Nisin, a bacteriocin produced during the exponential growth phase of *Lactococcus lactis* ATCC 11454, inhibits the growth of a broad range of Gram-positive bacteria. Gram-negative bacteria can also be inhibited by nisin with EDTA. In this study, nisin production was assayed by the agar diffusion method using *Lactobacillus sake* ATCC 15521 and a recombinant *Escherichia coli* DH5-α expressing the recombinant green fluorescent protein as the nisin-susceptible test organisms. The titers of nisin expressed and released in culture media were quantified and expressed in arbitrary units (AU/mL of medium) and converted to standard nisin concentration (Nisaplin®, 25 mg of pure nisin with an activity of 1×10^6 AU/mL). The expression and release of nisin by *L. lactis* in skimmed milk (9.09% total solids) with Man Rugosa Shepeer-Bacto Lactobacilli broth (1:1) was monitored in a 5 L New Brunswick fermentor. Combining EDTA with nisin increased the bactericidal effect of nisin on the bacteria examined. The presence of EDTA was necessary to inhibit *E. coli* growth with nisin. *L. sake* was shown to be a good indicator for the evaluation of nisin release in the culture media, including with the addition of EDTA.

Index Entries: Nisin; *Lactococcus lactis; Lactobacillus sake;* EDTA; *Escherichia coli;* recombinant green fluorescent protein.

Introduction

Nisin, a naturally occurring antimicrobial polypeptide discovered in 1928 *(1,2)*, is a monomeric pentacyclic subtype A lantibiotic peptide (3,353 Da) synthesized by *Lactococcus lactis* ssp. *lactis* *(3,4)* during exponential

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growth *(5,6)*. Nisin and other subtype A lantibiotics are characterized by a strong bactericidal activity by inhibiting spore germination *(7)* and the growth of many strains of Gram-positive bacteria *(8)*. Nisin is used as a natural preservative in food and dairy industries and is approved by the US Food and Drug Administration and has GRAS (generally regarded as safe) status *(9)*. This bacteriocin has been also applied in dental care products *(10)*, and in pharmaceutical products for the potential use as a contraceptive and for the treatment of stomach ulcers and colon infections *(11–13)*. Nisin solubility and stability increase substantially with increasing acidity. Nisin is stable at pH 2.0 and can be autoclaved at 121°C *(14)*. The liberation of nisin from cells into the propagating medium is dependent on the pH of the medium. At pH < 6.0, at least 80% of expressed nisin is released into the growth medium. On the other hand, when the bacterium *L. lactis* is cultivated in pH > 6.0, the majority of the nisin is retained within the cell membrane or intracellularly *(15)*. Nisin is not generally active against Gram-negative bacteria, yeasts and fungi. The outer membrane of Gram-negative bacteria prevents nisin from reaching the site of action. Outer membrane permeability can be altered by treatment with chelators, such as EDTA (disodium ethylenediamine tetraacetate), or high hydrostatic pressure, resulting in increased sensitivity toward nisin. *(16–22)*. The recombinant green fluorescent protein (GFPuv), expressed by *Escherichia coli* DH5-α, is widely utilized as a genetic protein marker. GFPuv in the cells can be easily visualized by a UV hand lamp making this a versatile tool for a variety of biotechnological applications and as a potential biological indicator in the preservation of manufactured and processed products *(23)*. In a system combining different antimicrobials, treatment with nisin/EDTA or nisin/potassium sorbate at 10°C exhibited significant population growth inhibition of *E. coli* O157:H7 compared with samples treated with nisin, EDTA or potassium sorbate alone *(19,24)*. Vessoni Penna and Moraes (2002) *(6)* studied the production of nisin by *L. lactis* ATCC 11454 in Man Rugosa Shepeer-Bacto Lactobacilli (MRS) broth. Varying concentrations of sucrose (5–12.5 g/L), asparagine (7.5–75 g/L), potassium phosphate (6–18 g/L), and Tween-80 (1–6.6 g/L) were added to MRS broth to determine which additive was most influential on growth rates, nisin expression and release into the media.

The authors observed a positive correlation between nisin production and biomass of *L. lactis* dependent on the balance of sucrose and asparagine, but independent of potassium phosphate. In a previous study *(25)*, culture media with milk was observed to provide better conditions for *L. lactis* growth and its concomitant expression of nisin. Milk alone (9.09% total solids) favored nisin expression and release into the media for all five transfers, from 0.1 to 0.4 g/L, similar to that attained at the first transfer for both 25% MRS plus 25% milk and 25% M17 plus 25% milk, with the latter media providing the highest nisin concentration, 3.6 g/L, after the fifth transfer. The effects of a milk-based medium on nisin

expression by *L. lactis* were investigated further to analyze growth conditions in a fermentor, compared with batch culture conditions.

The inhibitory activity of nisin on Gram-negative organisms can be improved by combining nisin with EDTA in culture media *(26)*. The detection of expressed nisin was evaluated by the diffusion method in agar using two susceptible bacteria, *Lactobacillus sake* (Gram-positive), and *E. coli* (Gram-negative). To evaluate the bactericidal activity of nisin on Gram-negative bacteria, EDTA was added to the samples or standard nisin solution.

Material and Methods

The nisin-producing strain of *L. lactis* ATCC 11454, the nisin-susceptible indicator strains of *L. sake* ATCC 15521 and a recombinant *E. coli* ATCC DH5-α were used in this study. The cultures of *L. lactis* and *L. sake* were maintained at –80°C in MRS broth (Difco®, Detroit, MI) with 50% (v/v) of glycerol. *E. coli* was grown (24 h/100 rpm/37°C) in Luria Bertani broth (LB) (Difco) supplemented with 100 µg/mL ampicillin (amp) to maintain a plasmid, with cultures stored at –80°C with 50% (v/v) of glycerol, following previously published procedures *(23,25)*.

Microorganism and Inoculum Preparation

Before inoculating experimental media, 100 µL of the stock culture of *L. lactis* were added to 50 mL of MRS broth (Difco) in 250-mL flasks (preinoculum) and incubated for 36 h/100 rpm/30°C. From the preinoculum, 5 mL aliquots of the cell suspension were transferred into 50 mL of experimental medium (MRS broth plus skimmed milk, 1:1) in 250-mL flasks (first transfer) and incubated for another 36 h (100 rpm/30°C). Cultures were transferred five times (100 rpm/30°C/36 h) using 5 mL aliquots of broth culture for each new volume of the experimental medium in accordance with previous studies *(25)*; only the preinoculum and the first and second transfers into MRS plus skimmed milk (1:1) were subsequently used for the fermentor cultures in this work.

Single Batch Fermentation

A 15 mL aliquot of the first transfer culture was mixed into 150 mL of culture medium (75 mL MRS + 75 mL skimmed milk) and incubated at 36 h/100 rpm/30°C (second transfer culture). The entire 150 mL of this second transfer culture was poured into 1.5 L of the same medium (MRS plus skimmed milk, 1:1, pH = 6.25–6.31) in a 5 L bench-scale fermentor (NBS-MF 105, New Brunswick Scientific, New Brunswick, NJ). The initial cell concentration in the fermentor was 0.58 ± 0.10 g/L. The total incubation time was 16 h at 30°C to observe variations of nisin expression associated with growth conditions. Foaming was controlled as needed by adding 0.5 mL of dimethylpolysiloxane (Sigma-Aldrich, St. Louis, MO). Agitation and aeration were 200 rpm and 0.5 vvm, respectively. The airflow was

measured by an on-line rotameter and set using a needle valve. The pH of the medium during cultivation was measured by an electrode (Ingold, Woburn, MA). Before the addition of inoculum to the fermentor, the propeller speed, aeration rate, and the temperature (30°C) were adjusted.

Analytical Procedures

After every 36 h incubation period, 10 mL of cell suspensions were aseptically withdrawn from the flasks and tested for pH, cellular density, colony number, and nisin concentrations. For this study, each fermentor culture was performed in triplicate. The pH measurements were performed in 10 mL of culture suspension with a Accumet AR20 pH/mV/conductivity meter (Fisher Scientific, Hampton, NH) calibrated with standard buffer solutions (Merck; Whitehouse Station, NJ) pH = 4.0, 7.0, 10.0, at 25°C. The cellular biomass concentration, expressed in mg of dried cellular weight per liter of broth (mg DCW/L), was determined from the optical density at 660 nm (OD_{660}) in a 1 cm path length quartz cuvet in a spectrophotometer (Beckman DU-600, CA, USA). 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®, SP, Br). The equation for the calibration curve ($R^2 = 0.998$) was given by:

$$OD_{660} = 0.0145 + 0.0022 \times DCW \text{ or}$$
$$DCW\ (mg/L) = [(OD_{660}) - 0.0145)]/(0.0022)$$

Culture populations were assayed by the plate count method expressed in colony forming units (CFU) per mL of broth (CFU/mL) in plate count agar (Difco) at 30°C for 24 h. Cell numbers were related to the reference curve associating OD_{660} to dry cell weight (mg DCW/L) of the same suspension, where $OD_{660} = 0.01$ was equivalent to 10^4 CFU/mL.

Nisin Activity Detection

As done in previous work *(25)*, the pH of the cell suspensions of *L. lactis* was adjusted from pH = 6.0 to pH = 4.0 and from pH = 4.0 to pH = 2.5 with 0.2 *N* HCl. The pH adjustment, before centrifugation, was done to coagulate the milk, releasing nisin into a clear supernatant (after centrifugation) to facilitate nisin detection.

For nisin activity detection, following methods of previous works *(6,24,25)*, the titers of nisin expressed and released in culture media were quantified and expressed in arbitrary units (AU/mL of medium) by the agar diffusion assay utilizing *L. sake* ATCC 15521 (Gram-positive) and a recombinant *E. coli* ATC DH5-α (Gram-negative) as nisin-susceptible indicator strains.

L. sake was grown in 50 mL of MRS broth in 250-mL flasks and incubated (24 h/100 rpm/30°C). A 1.5 mL aliquot of the suspension ($OD_{660} = 0.4$) was transferred and mixed with 250 mL of soft agar (MRS broth with 0.8%

of bacteriologic grade agar) in 500-mL flasks. Each 20 mL of inoculated medium was transferred to Petri plates (100 mm dia).

E. coli was grown in 50 mL of LB in 250-mL flasks and incubated (24 h/100 rpm/37°C) supplemented with 100 μg/mL amp. An aliquot of the suspension was transferred onto the surface of LB-amp agar-soft with isopropyl-β-D-thiogalactopyranoside (IPTG) added to a final concentration of 0.5 m*M* (w/v) and incubated at 37°C. Another aliquot of the suspension was serially diluted in physiologic buffer into 1:1000 dilution corresponding to an $OD_{660} = 0.005$. A 0.6 mL aliquot of this diluted suspension ($OD_{660} = 0.005$) was transferred and mixed with 100 mL of soft agar (LB broth with 100 μg/mL amp, 375 μL of IPTG and 0.8% of bacteriologic grade agar) in 250-mL flasks. Each 20 mL of inoculated medium was transferred to Petri plates. The recombinant *E. coli* was grown with amp and IPTG added to the media to maintain the plasmid and express GFP, conditions that will be used in future studies utilizing fluorescence detection.

From every fermentor culture, 50 μL of culture supernatant from centrifuged *L. lactis* suspension, with or without 7.45 mg/mL EDTA, was transferred into each of four wells on the surface of the *L. sake* inoculated agar. With culture supernatant mixed previously with 7.45 mg/mL EDTA or without, 50 μL of this mixture was transferred into each of four wells on the surface of the *E. coli* inoculated agar.

The relation between (AU/mL) and international units (IU/mL) was determined by using Nisaplin (a commercial purified nisin preparation containing 25 mg of nisin per gram of Nisaplin, corresponding to 10^6 IU/g Nisaplin; Aplin & Barret, Sigma Chemical); and a standard solution of nisin (Nisaplin), containing 250 μg of nisin per mL corresponded to 10^4AU/mL. IU conversions are included in this work to correlate this work with past references *(24,25)*.

With *E. coli* as the susceptible bacteria, standard nisin was diluted in either deionized water or in 0.02*N* HCl, with a final concentration of 0.75% (w/v) NaCl, and 7.45 mg/mL added EDTA. For comparison with the sensitivity of *L. sake* to nisin mixed with EDTA, another standard curve was generated.

The activity of nisin expressed in AU/mL was converted for nisin in milligrams per milliliters (mg/mL), through the relation:

$$\text{Nisin (mg/mL)} = \frac{(z \times 0.025)}{1000}, \text{ where } z = \text{AU/mL}$$

Results and Discussion

Susceptible Bacteria and the Association of Nisin With EDTA

Using *E. coli* to evaluate the activity of nisin in solutions with 7.45 mg/mL EDTA (Table 1), the zones of inhibition ranged from 10 mm to 14.75 mm, which correlated to 10 to 10^5 AU/mL of nisin in acidified solution

Table 1
Standard Curves and Conversion Equations Associating the Inhibition Halos (H, mm, Zones Lacking *E. coli* or *L. sake* Growth) With the Standard Nisin Arbitrary Units (10–10^5 AU/mL)

			Standard nisin activity (AU/mL)					Conversion equation	
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}		
Strains	Solutions	pH	Diameters of inhibition halos (SD = ±0.5 mm)					AU/mL	R^2
E. coli	Nisin plus EDTA in 0.02*N* HCl	2.5	10	12.5	13.25	14.5	14.75	$10^{(0.783 \times H - 7.1011)}$	0.94
	Nisin plus EDTA in H_2O	4.0	13.3	14.8	15.7	16.25	17	$10^{(1.0693 \times H - 13.456)}$	0.96
L. sake	Nisin plus EDTA in 0.02*N* HCl	2.5	8.5	11.5	14.75	16.75	17.5	$10^{(0.4102 \times H - 2.6612)}$	0.95
	Nisin plus EDTA in H_2O	4.0	13	14	16.25	17.25	18.25	$10^{(0.7097 \times H - 8.1774)}$	0.97
	Nisin in skimmed milk	4.0	10	15.25	19.25	24.5	26.75	$10^{(0.2307 \times H - 1.4174)}$	0.98
	Nisin in skimmed milk plus EDTA	4.0	5	17	19.8	22.31	23.56	$10^{(0.2639 \times H - 0.3641)}$	0.98
	Nisin in skimmed milk	6.0	10.75	14	18.5	21	22.25	$10^{(0.3211 \times H - 2.5553)}$	0.96
	Nisin in skimmed milk plus EDTA	6.0	8.75	14.5	16.75	20.5	20.75	$10^{(0.3065 \times H - 1.9808)}$	0.92

Observation: the calibration curves between AU/mL and IU/mL, 1.09 ± 0.17 AU corresponded to 1.0 IU (40 IU = 1 μg of pure nisin A).

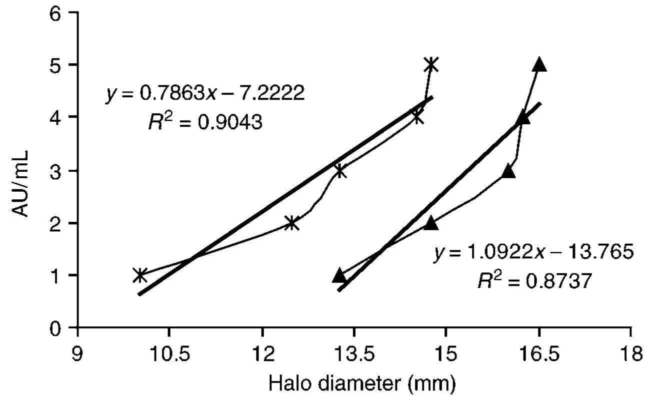

Fig. 1. Standard curve relating the action of nisin plus EDTA in [-✳-] acidified solution with HCl (pH 2.5) and [-▲-] solutions (pH 4.0) utilizing *E. coli* cells.

(pH 2.5). Nisin plus EDTA in aqueous solution (pH 4.0) gave zones of 13.3–17 mm corresponding the nisin activity of 10 to 10^5 AU/mL equivalent a concentration of 0.025 µg/mL to 2500 µg/mL. The relation between both slopes (1.0693 and 0.783) of the standard curves provided 37% higher arbitrary units on the nisin activity when nisin was diluted and mixed with EDTA in aqueous solution (pH 4.0) compared with the solution (pH 2.5) adjusted with 0.02*N* HCl. The acidified solution reduced the nisin activity on the susceptibility of the *E. coli* growth in agar (Fig. 1)

Using cells of *L. sake* to evaluate the activity of standard nisin in solutions of 7.45 mg/mL EDTA, (i) the zones of inhibition ranged from 8.5 mm to 17.5 mm correlated the nisin activity of 10 to 10^5 AU/mL, equivalent a concentration of 0.025 µg/mL to 2500 µg/mL from a conversion equation: Log AU/mL = 0.4102 × H – 2.6612, relating the action of nisin and EDTA in acidified solution with HCl (pH 2.5); (ii) the zones of inhibition ranged from 13 mm to 18.25 mm correlated the nisin activity of 10 to 10^5 AU/mL, equivalent a concentration of 0.025 µg/mL to 2500 µg/mL from a conversion equation: Log AU/mL = 0.7097 × H – 8.1774, relating the action of nisin plus EDTA in water (pH 4.0). The relation between both slope (0.4102 and 0.7097) of the standard curves provided 73% higher arbitrary units for nisin when diluted and mixed with EDTA in water (pH 4.0) compared with the acidified solution to pH 2.5 with 0.02*N* HCl.

The low pH in acidified solution (pH 2.5) lowered nisin susceptibility of *E. coli* and *L. sake*, and was verified previously by the authors *(25)*, but growth was not inhibited by a nisin-free HCl solution (pH 2.5) added to the wells for *L. sake*, *E. coli*, and *L. sake* susceptibility in nisin solutions with EDTA adjusted to pH 2.5 exhibited higher variability, around 20% among halos, for the same AU/mL than with nisin with EDTA at pH 4.0 with variability around 4% (Table 2). Examining Tables 1 and 2, it can be noted that the association of EDTA and nisin has shown to increase the bactericidal effect on *E. coli*, the Gram-negative nisin-susceptible bacteria. *L. sake* has been

Table 2
Inhibitions Halos (mm), Zones Lacking *L. sake* or *E. coli* Growth, Converted to Nisin Arbitrary Units Per Milliliter (AU/mL) From the Placement of 50 µL of Either Supernatant (Centrifuged Samples From the Fermentor) or Standard Nisin Solutions, Mixed With 7.45 mg/mL EDTA Diluted in Water or in 0.02*N* HCl, Plus 0.75% NaCl Into the Wells on the Surface of Inoculated Agar (Diffusion Assay)

		L. sake	Sensitive strain for nisin activity		
		Halos	Conversion equation		
Supernatant from fermentor	pH	*M*m	AU/mL	AU/mL	Log_{10}
With EDTA in water (1:1)	4.0	16	$10^{(0.7097 \times H - 8.1774)}$	1505	3.18
With EDTA in 0.02*N* HCl (1:1)	2.0	11.5	$10^{(0.4102 \times H - 2.6612)}$	114	2.06
With EDTA	4.8	13.75	$10^{(0.7097 \times H - 8.1774)}$	38	1.58
Untreated (without EDTA)	4.8	12.25	$10^{(0.33211 \times H - 2.5553)}$	33	1.51
		E.coli	Sensitive strain for nisin activity		
		Halos	Conversion equation		
Supernatant from fermentor	pH	*M*m	AU/mL	AU/mL	Log_{10}
With EDTA in water (1:1)	4.0	16	$10^{(1.0693 \times H - 13.456)}$	4495	3.65
With EDTA in HCl (1:1)	2.0	0	$10^{(0.783 \times H - 7.1011)}$	nd	
With EDTA	4.8	13	$10^{(0.783 \times H - 7.1011)}$	11	1.05
Untreated (without EDTA)	4.8	0	$10^{(0.783 \times H - 7.1011)}$	nd	

		E. coli	Sensitive strain for nisin activity		
		Halos	Conversion equation		
Standard nisin solution	pH	mm	AU/mL	AU/mL	Log_{10}
50 μg/mL nisin (2×10^3 AU/mL) In water	4.0	15.75	$10^{(1.0693 \times H - 13.456)}$	2429	3.39
50 μg/mL nisin (2×10^3 AU/mL) in HCl	2.0	13	$10^{(0.783 \times H - 7.1011)}$	1197	3.08
50 μg/mL nisin (2×10^3 AU/mL) In HCl : water (1:1)	2.0	13	$10^{(0.783 \times H - 7.1011)}$	1197	3.08
250 μg/mL nisin (1×10^4 AU/mL) In water	4.0	17	$10^{(1.0693 \times H - 13.456)}$	52,735	4.72
250 μg/mL nisin (1×10^4 AU/mL) In HCl	2.0	13.5	$10^{(0.783 \times H - 7.1011)}$	2947	3.47
250 μg/mL nisin (1×10^4 AU/mL) In HCl : water (1:1)	2.0	14.8	$10^{(0.783 \times H - 7.1011)}$	30,711	4.49

nd, none detected.

confirmed to be a sensitive and reliable Gram-positive biological indicator for the detection of nisin, even without the addition of EDTA.

It was also observed that the formation of a greater inhibition zone of *L. sake* growth, around 45%, occurred with nisin plus EDTA in water rather than in acidified solution (pH 2.5), with a nisin activity to 10 AU/mL equivalent a concentration of 0.025 μg/mL (Table 1). On the other hand, for *E. coli* with nisin plus EDTA, the inhibition zone was 30% greater in water (nisin activity to 10 AU/mL equivalent a concentration of 0.025 μg/mL) than in acidified solution. The pH between 4.0 and 6.0 of skimmed milk showed a variation in the susceptibility of *L. sake* to nisin of around 20% (Table 1). Treatment with nisin solution without EDTA had no effect on the growth of *E. coli*. Only the supernatant, from centrifuged culture samples, treated with 7.45 mg/mL EDTA and diluted (1:1) in water were significantly different ($p < 0.05$) when compared with the untreated samples (Table 2). On the other hand, EDTA showed no influence on *L. sake* growth inhibition. For both strains *E. coli* and *L. sake*, mixing EDTA in water and then adding to the supernatant (1:1) seemed to enhance cell sensitivity and result in larger inhibition zones compared with wells applied with EDTA directly mixed into the supernatant. The mixing of EDTA in water and then adding to the supernatant (1:1) showed equal sized halos for *L. sake* and *E. coli* on the inoculated agar, corresponding to nisin activity of 1.51×10^3 AU/mL and 4.5×10^3 AU/mL, equivalent to a nisin concentration of 38 μg/mL and 113 μg/mL, respectively. For EDTA mixed directly into the supernatant, the inhibition halos of *E. coli* and *L. sake* growth were also similar, 13.75 mm and 13 mm, respectively, corresponding to 11 AU/mL and 38 AU/mL, with similar activity to 33 AU/mL nisin in the untreated supernatant.

The influence of EDTA on *E. coli* susceptibility was confirmed and diluting EDTA in water before mixing to the supernatant was significant. This susceptibility performance has been also confirmed by *E. coli* cells to evaluate the standard nisin activity at concentrations of 50 and 250 μg/mL (2×10^3 AU/mL and 10^4 AU/mL) in solutions with added EDTA. The zones of inhibition were greater for solutions mixed with EDTA diluted prior to mixing than directly added to the standard nisin solution and ranged from 15.75 mm to 17 mm, corresponding in nisin activity to 2.4×10^3 AU/mL and 5.27×10^4 AU/mL, equivalent to a nisin concentration of 60 μg/mL and 1318 μg/mL and dropped to 13 mm and 13.5 mm, respectively corresponding in nisin activity to 1.2×10^3 AU/mL and 2.9×10^3 AU/mL equivalent to a nisin concentration of 20 μg/mL and 73 μg/mL, 10 times lower than the former values obtained with EDTA diluted in water before adding to the nisin solution. Further work needs to be done to determine the basis of this discrepancy caused by mixing or direct addition of EDTA; this may be related to differences in the solubilization of EDTA under these conditions.

It has been claimed that the bactericidal effect of nisin activity on Gram-negative organisms can be improved when used in combination with other antimicrobial agents such as chelators *(19,25)*. Cutter and

Siragusa (1995) *(25)* reported that nisin together with chelators exhibit a better antimicrobial activity on Gram-negative pathogens in culture media. However, this combination was found to be less effective when applied to real food systems, compared with culture media. Fang and Tsai (2003) *(19)* studied the inhibitory effect of antimicrobials and the combination of antimicrobials with EDTA over *E. coli* O157:H7 in ground beef at 10°C. They observed that the association of nisin (10^3 IU/mL) and EDTA (7.45 mg/mL) mixed to the ground beef samples (added with or without 1.5% $CaCl_2$) showed significant growth inhibition of *E.coli* compared with the samples treated with nisin or EDTA alone. The effect of the association of EDTA-nisin was similar in our experiments and confirmed our results.

We verified that the presence of EDTA was essential to improve nisin activity on *E. coli* growth inhibition. However, *L. sake* was more sensitive for the evaluation of nisin release in the culture media, even with nisin and EDTA in the supernatant. The mechanism of growth inhibition by EDTA is not fully understood, but generally attributed to its chelating activity. EDTA binds primarily divalent cations *(27)* that are present in the supernatant obtained from the growth media. Therefore, we can imply that washing the cells should be enough to extract the majority of salts from the culture media, in order not to limit the action of EDTA to destabilize the membrane of some Gram-negative strains by chelating calcium and magnesium, which are necessary for LPS to bind to the cell wall *(27–29)*. It is possible that the addition of EDTA can cause calcium and magnesium deprivation of the cells or EDTA may be toxic to the cells by some other mechanism.

It was observed that the formation of a larger inhibitory halos occurred when EDTA and nisin were diluted in water before mixing, at $pH = 4.3 \pm 0.5$. In all the samples, for both sensitive bacteria, the dilution in aqueous solution with HCl (typically used to dissolve nisin) to $pH = 2.5$ provided smaller halo diameters when compared with the halos formed using water plus EDTA. This may be related to the pKa of EDTA and its effectiveness as a chelator.

Branen and Davidson (2004) *(30)* confirmed that EDTA enhanced the synergetic activity of nisin, monolaurim, and lysozyme in tryptic soy broth against two enterohemorrhagic *E. coli* strains, but not against *Salmonella enteritidis* or *P. fluorescens*. However, the authors observed that none of these antimicrobial combinations with EDTA was shown particularly effective in ultra high temperature milk held at 25°C against the same strains of *E. coli*, demonstrating that the activity of antimicrobials in a food system may be affected by a number of factors, as well as fat, protein, salt concentration, and storage temperature of the samples. Gill and Holley (2003) *(23)* found a probability of 89%, 73%, and 43% of two-agent inhibitory (EDTA plus chrisin) interactions against, respectively, *S. typhimurium*, *E. coli, and Serratia grimessi* on growing cells in nutrient broth with NaCl and nitrite added. Those findings reinforce the association of EDTA-nisin in water exhibiting significant synergetic activity on *E. coli* cells than in the

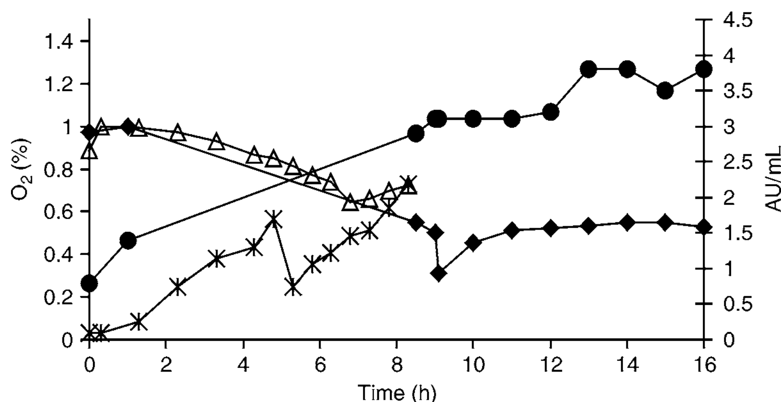

Fig. 2. Oxygen consumption and nisin activity of the first and second fermentations (●1° inoculum AU/mL; ♦1° inoculum $\%O_2$; ✳ 2° inoculum AU/mL; Δ 2° inoculum $\%O_2$). For media with 50% skimmed milk plus 50% MRS, fermentor cultures was started from the first transfer cultures, the relation between AU/mL and inhibition halo (H, mm) was calculated through the equation: $AU/mL = 10^{(0.2307 \times H - 1.4174)}$; and for the second transfer culture, the relation between AU/mL and inhibition halo (H, mm) was through the equation: $AU/mL = 10^{(0.3211 \times H - 2.5553)}$.

MRS plus skimmed milk (1:1), suggesting no interference of excess salts on the EDTA-nisin interaction.

Single Batch Fermentation

For 50% skimmed milk mixed with 50% MRS, extending the incubation to 16 h of fermentation growth for *L. lactis* resulted in higher expression of nisin, with titers of 5934 AU/mL, four times greater than the nisin titer of 1377 AU/mL obtained with 8 h fermentation, with a constant 50–55% O_2 demand and pH between 4.5 ± 0.5 (Fig. 2). Up to now, experiments were carried out with inoculum from the first and second transfer cultures for 8 h and 16 h fermentations, respectively. The data obtained from fermentation conditions were 22 times lower than the titer values obtained from *L. lactis* nisin expression grown in 25% milk and 25% MRS (with the fourth transfer culture as inoculum) in rotary shaker (100 rpm) for 36 h (at 30°C), observed from a previous study *(24)*. These results *(24)* indicate that the fermentation growth conditions could be improved with culture medium (25% milk and 25% MRS) inoculated with the third, fourth, and fifth transfer cultures of *L. lactis*. Although the expression of nisin was observed throughout fermentation, the constant increase of nisin expression observed with 8 h fermentation was coincident to the stabilization of O_2 consumed, in which the O_2 concentrations were adjusted to cell growth.

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