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Nisin Production Utilizing Skimmed Milk Aiming to Reduce Process Cost

Angela Faustino Jozala, Maura Sayuri de Andrade, Luciana Juncioni de Arauz, Adalberto Pessoa Jr., and Thereza Christina Vessoni Penna*

Department of Biochemical and Pharmaceutical Technology, School of Pharmaceutical Science, University of São Paulo, SP, Brazil, E-mail: tcvpenna@usp.br

Abstract

Nisin is a natural additive for conservation of food, pharmaceutical, and dental products and can be used as a therapeutic agent. Nisin inhibits the outgrowth of spores, the growth of a variety of Gram-positive and Gram-negative bacteria. This study was performed to optimize large-scale nisin production in skimmed milk and subproducts aiming at low-costs process and stimulating its utilization. *Lactococcus lactis* American Type Culture Collection (ATCC) 11454 was developed in a rotary shaker (30°C/36 h/100 rpm) in diluted skimmed milk and nisin activity, growth parameters, and media components were also studied. Nisin activity in growth media was expressed in arbitrary units (AU/mL) and converted to standard nisin concentration (Nisaplin®, 25 mg of pure nisin is 1.0×10^6 AU/mL). Nisin activity in skimmed milk 2.27 $g_{\text{total solids}}$ was up to threefold higher than transfers in skimmed milk 4.54 $g_{\text{total solids}}$ and was up to 85-fold higher than transfers in skimmed milk 1.14 $g_{\text{total solids}}$. *L. lactis* was assayed in a New Brunswick fermentor with 1.5 L of diluted skimmed milk (2.27 $g_{\text{total solids}}$) and airflow of 1.5 mL/min (30°C/36/200 rpm), without pH control. In this condition nisin activity was observed after 4 h (45.07 AU/mL) and in the end of 36 h process (3312.07 AU/mL). This work shows the utilization of a low-cost growth medium (diluted skimmed milk) to nisin production with wide applications. Furthermore, milk subproducts (milk whey) can be exploited in nisin production, because in Brazil 50% of milk whey is disposed with no treatment in rivers and because of high organic matter concentrations it is considered an important pollutant. In this particular case an optimized production of an antimicrobial would be lined up with industrial disposal recycling.

Index Entries: Artificial compounds; EDTA; fermentation processes; Gram-negative; Gram-positive; *Lactococcus lactis*; nisin.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

Nisin, a naturally occurring antimicrobial polypeptide, discovered in 1928 *(1,2)*, is a monomeric pentacyclic subtype A antibiotic peptide (3.35 kDa with 34 amino acid residues), synthesized by *Lactococcus lactis* subsp. lactis *(3,4)* during exponential phase of bacteria growth *(5,6)*. Nisin is used as a natural preservative in food and dairy industries, approved by Food and Drug Administration and GRAS *(7)*, meeting the requirements of safe food with fewer chemical additives.

Applications of nisin include dental-care products *(8)*, pharmaceutical products such as stomach ulcers and colon infection treatment and potential birth control *(9–11)*. Nisin solubility and stability improves substantially with a decrease in pH values. Nisin is stable at pH 2.0, insoluble at pH 8.5, and can be autoclaved at 121°C without denaturation *(12)*. The complete inactivation of nisin activity is observed after 30 min at 63°C and pH 11.0 *(13)*.

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 disodium ethylenediamine tetraacetate (EDTA) or high hydrostatic pressure, resulting in increased sensitivity toward nisin *(14–20)*. The mechanism of growth inhibition by EDTA is not fully understood, but generally attributed to its chelating activity. EDTA binds primarily divalent cations *(21)* that are present in the supernatant obtained from the growth media, which salts decrease the amount of EDTA added. Therefore, we can imply that the washing of the cells should be enough to extract the majority of salts from the culture media, in order not to compete with EDTA main activity of destabilizing the membrane of some Gram-negative strains by chelating Ca and Mg salts, which are necessary for lipopolysaccharide to bind to cell wall *(21–23)*.

In a system combining different antimicrobials, treatment with nisin/EDTA or nisin/potassium sorbate at 10°C showed a meaningful inhibition in *Escherichia coli* O157-α5 compared with samples treated with nisin, EDTA, or potassium sorbate alone *(17,25)*. The inhibitory activity of nisin on Gram-negative organisms can be improved by combining nisin with EDTA in culture media *(26)*. Vessoni Penna et al. *(27)* using *L. lactis* ATCC 11454 observed that nisin production was the highest in a growth medium containing 25% skimmed milk added to either 25% M17 or MRS, it was showed that nisin production depend on the nutrients concentration and the transfers renewed the media each at 36 h. The influence of milk compounds on nisin activity was observed in previous work *(28)* and skimmed milk (9.09% dry matter) increased nisin activity and release into the media for all five transfers. Although the formulations of skimmed milk diluted with MRS broth were found to stimulate optimal nisin production.

Lactic acid bacteria are fastidious microorganisms and require a medium containing nutrients, which enhance the growth and production of nisin *(29)*.

In this study, diluted skimmed milk in different concentrations was used to improve nisin production and also examined the utilization of skimmed milk compounds (artificial reproduction) to determine which nutrient is essential to nisin production. With nisin activity related to growth conditions of *L. lactis*, the effects of culturing parameters such as media components were evaluated in this study to optimize the expression of nisin and release into media.

Material and Methods

The nisin-producing strain of *L. lactis* ATCC 11454 and the nisin-sensitive indicator strain of *L. sake* ATCC 15521 (Gram-positive) were used in this study. The cultures of *L. lactis* and *L. sake* were maintained at –80°C in MRS broth (Man Rugosa Shepeer-Bacto Lactobacilli MRS broth, DIFCO) with 40% (v/v) of glycerol *(26–28).*

Growth Medium and Inoculum

The influence of milk components on nisin activity was studied in previous work *(27,28).* In this present work different medium were elaborated with diluted skimmed milk to improve the growth conditions for *L. lactis*. Before inoculating experimental media, 100 µL of the stock culture of *L. lactis* was grown (preinoculum) in MRS broth (DIFCO) into 50 mL of broth in 250-mL Erlenmeyer flasks and incubated on a rotary shaker (100 rpm) at 30°C for 36 h. From the growth culture, 5-mL aliquots of bacterial suspension were transferred to 50 mL of the experimental medium in 250-mL flasks, which were incubated for another period of 36 h (100 rpm/30°C). The transfer and incubation of a new volume of each medium was repeated five times (first, second, third, fourth, and fifth transfers).

In the first group of assays, utilized skimmed milk (9.09 $g_{total\ solids}$, standard concentration) was developed with following experimental medium: (a) skimmed milk at 50% of standard concentration (4.54 $g_{total\ solids}$); (b) skimmed milk at 25% of standard concentration (2.27 $g_{total\ solids}$); (c) skimmed milk at 12.5% of standard concentration (1.14 $g_{total\ solids}$). All medium was diluted in sterile distillated water (Table 1).

In Second group of assays, utilized skimmed milk compounds (artificial reproduction) was developed with following experimental medium: (a) casein (0.75 g) and lactose (1.25 g); (b) casein (0.75 g), lactose (1.25 g) plus calcium (0.06 g); (c) casein (0.75 g), lactose (1.25 g) plus sodium citrate (0.01 g); and (d) casein (0.75 g), lactose (1.25 g), calcium chloride (0.06 g) plus sodium citrate (0.01 g) (Table 2).

Fermentation Process

Preinoculum was prepared with 100 µL of the stock culture of *L. lactis* and was grown into 150 mL of MRS broth (36 h/100 rpm/30°C). The entire 150 mL of this culture was poured into 1.5 L of the diluted skimmed milk

Table 1
Nisin Production, Specific Production, Productivity, Proteins, and Sugar Consumption of *L. lactis* Growth for Every Transfer After 36 h to Diluted Skimmed Milk

		Nisin production					Biomass	Productivity	Proteins	Sugars
	Transfers	pH	Halo (mm)	$10^{(0.2408x - 0.8745)}$ (AU/mL)	Log (AU/mL)	0.025 (mg/L)	$y = 2.1042x + 0.124$ (g_{DCW}/L)	mg_{nisin}.mg$_{DCW}$/h	$y = 0.9023x - 0.0329$ (g_{casein}/L)	$y = 0.537x - 0.0127$ ($g_{lactose}$/L)
Compounds	Preculture	4.5	17	1656.15	3.22	41.40	0.87	–	–	–
	1	4.8	11.75	90.14	1.95	2.25	0.47	–	17.67	21.53
	2	4.45	12.5	136.62	2.14	3.42	0.36	–	17.28	21.06
4.54 g and pH 6.8	3	4.5	15.5	720.94	2.86	18.02	1.37	–	14.04	21.52
	4	4.35	19	5019.96	3.70	125.50	1.43	0.0	14.86	22.74
	5	4.43	20	8739.77	3.94	218.49	0.94	0.01	11.98	18.45
	Preculture	4.55	17.5	2185.24	3.34	54.63	0.85	<0.01	–	–
	1	5.33	16.5	1255.16	3.10	31.38	0.49	<0.01	17.67	13.45
	2	5.63	17.5	2185.24	3.34	54.63	0.34	<0.01	17.28	13.16
2.27 g and pH 6.8	3	4.36	18.25	3312.07	3.52	82.80	1.58	<0.01	14.04	10.76
	4	4.51	18.75	4370.19	3.64	109.25	1.64	<0.01	14.86	11.37
	5	4.34	21.5	20077.05	4.30	501.93	1.78	0.01	11.98	9.22
	Preculture	4.55	12	103.54	2.02	2.59	0.84	–	–	–
	1	4.3	13.75	273.21	2.44	6.83	0.28	–	2.94	4.96
	2	5.75	11.25	68.31	1.83	1.71	0.09	–	8.87	2.22
1.14 g and pH 6.8	3	5.92	–	–	–	–	0.55	–	2.28	4.89
	4	6.47	9.5	25.89	1.41	0.65	0.41	–	3.70	4.18
	5	6.73	–	–	–	–	1.91	–	4.50	2.94

Table 2
Nisin Production, Specific Production, Productivity, Proteins, and Sugar Consumption of *L. lactis* Growth, for Every Transfer After 36 h to Artificial Compounds

		Nisin production					Biomass	Productivity	Proteins	Sugars
	Transfers	pH	Halo (mm)	$10^{(0.2408x - 0.8745)}$ (AU/mL)	Log (AU/mL)	0.025 (mg/L)	$y = 2.1042$ $x + 0.124$ (g_{DCW}/L)	mg_{nisin} .mg $_{DCW}$/h	$y = 0.9023$ $x - 0.0329$ (g_{casein}/L)	$y = 0.537$ $x - 0.0127$ ($g_{lactose}$/L)
Compounds	1	3.9	14.5	414.10	2.62	10.35	0.15	–	1.08	13.41
	2	6.29	9.75	29.74	1.47	0.74	0.42	–	8.32	16.19
Media A	3	6.14	–	–	–	–	1.56	–	10.46	19.79
	4	6.52	11.25	68.31	1.83	1.71	1.31	–	13.13	19.68
	5	6.35	–	–	–	–	1.50	–	15.26	11.05
	1	4.23	14.75	475.66	2.68	11.89	0.14	–	0.40	13.33
	2	6.14	10.5	45.07	1.65	1.13	0.20	–	8.73	15.45
Media B	3	6.27	–	–	–	–	0.26	–	9.91	15.05
	4	6.64	–	–	–	–	0.23	–	13.70	18.14
	5	6.43	–	–	–	–	0.19	–	14.80	10.50
	1	4.36	11.25	68.31	1.83	1.71	0.18	–	1.08	13.41
	2	6.61	10.5	45.07	1.65	1.13	0.15	–	8.32	16.19
Media C	3	6.26	–	–	–	–	0.20	–	10.46	19.79
	4	6.51	–	–	–	–	0.25	–	13.13	19.68
	5	6.73	–	–	–	–	0.23	–	14.70	10.50
	1	4.6	12.75	156.93	2.20	3.92	0.14	–	0.06	12.84
	2	6.11	11	59.47	1.77	1.49	0.15	–	17.01	18.14
Media D	3	5.9	–	–	–	–	0.19	–	6.53	16.29
	4	6.37	–	–	–	–	0.26	–	13.48	19.84
	5	6.43	–	–	–	–	0.24	–	14.75	10.21

(2.27 $g_{\text{total solids}}$, pH 6.8) in a 2-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 36 h at 30°C to observe variations of nisin activity associated with growth conditions. Foaming was controlled as needed by adding 0.5 mL of dimethylpolysiloxane (Sigma-Aldrich, Saint Louis, MO). Agitation and aeration were 200 rpm and 1.5 vvm, respectively. The airflow was measured by an online 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

Assays in rotator shaker in each transfer 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. Samples were aseptically withdrawn from fermentor with interval of 4 h (10 sample points) and were collected and tested for biomass, nisin activity, and nutrients consumed. For this study, each sample was performed in triplicate.

Biomass, Total Sugars, and Total Proteins

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}) by the calibration curve [biomass (mg · DCW/L) = $2.1042 \times OD_{660} + 0.124$, $R^2 = 0.998$], as described in the previous work *(26–28)*. The lactose concentration, expressed in gram of lactose per liter of broth (g/L) was determined from the samples in the optical density at 540 nm (OD_{540}) through colorimetric Somogyi-Nelson methodology *(30)*. The standard curve [lactose g/L = $(0.537 \times OD_{540\ nm})$ (0.0127], was developed for different concentrations (0.25–0.01 g/L) with standard lactose solution (Merk, Darmstadt, Germany).

The protein concentration, expressed in gram of casein per liter of broth (g/L) was determined from the samples in the optical density at 660 nm (OD_{660}) through colorimetric Folin-phenol methodology described by Lowry *(31)*. The standard curve [casein g/L = $(0.9023 \times OD_{660\ nm})$ –0.0329] was developed for different concentrations (0.25–0.01 g/L) with the standard casein solution (Sigma, St. Louis, MO).

Nisin Activity

For nisin activity detection, the cell suspension was centrifuged at 12,000 rpm for 10 min at 25°C 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/mL of medium) by the agar diffusion assay *(6,22)* utilizing *L. sake* as a sensitive indicator microorganism. *L. sake* was grown in MRS broth and incubated (100 rpm/30°C/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% w/v of bacteriological grade agar). Each 20 mL of inoculated medium was transferred to Petri plates (100-mm diameter). After the agar solidified, 3-mm wells were cut out with a sterile metal pipe with 5 mm total diameter. The relation between (AU/mL) and international units (IU/mL) was determined by using Nisaplin® (a commercial purified nisin preparation containing 2.5 mg of nisin per gram of Nisaplin, corresponding to 10^6 IU/g Nisaplin; Aplin & Barret Ltd, Beaminster, UK, distributed by Sigma Chemical). Standard solution of nisin were prepared by dissolving 1 g of Nisaplin into 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 nisin solution were made as necessary by diluting in 0.02 *N* HCl and water. With the standard curve (AU/mL = $10^{0.2408\ H-0.8745}$), the concentrations of standard nisin (10^0–10^5 AU/mL) 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 mL (10^0–10^5 AU/mL). Based on the calibration curves between AU per mL and IU per mL, 1.09 ± 0.17 AU corresponded to 1.0 IU (40 IU = 1 μg of pure nisin A).

Using the standard solutions for calibration of nisin activity in all the assays, 10^6 AU of nisin corresponded to 0.025 μg_{nisin}/mL. The activity of nisin expressed in AU/mL was converted to nisin in milligrams per milliliters (mg/mL), through the relation: Nisin (mg/L) = ($z \times 0.025$), where z = AU/mL. The concentration of nisin was also expressed in milligrams per liters (mg/L); and in the production of nisin (mg/L/h), the formation of nisin in milligrams per liters related to incubation time (h). The specific production of nisin (mg/mg) is the ratio between nisin concentration (mg/L) and the dry weight cell (mg · DCW/L). Productivity was expressed in milligrams nisin per milligram of DCW per hour as the ratio of the hourly milligrams of nisin (mg/L/h) and biomass (DCW).

Results and Discussion

L. lactis was transferred consecutively five times in the same growth medium and incubated under the same conditions (100 rpm/30°C/36 h) as proceeded in our previous works *(26–28)*. Tables 1 and 2 show the results for nisin activity (AU/mL) and concentration (mg/L) in the analyzed samples.

Dilution of Milk

Vessoni Penna et al. *(27)* observed that milk at standard concentration (9.09% dry matter) increased nisin activity and released it into the media

Table 3
Compounds of Skimmed Milk in Different Dilutions

Nutrients	50 mL	25 mL	12.5 mL	6.25 mL
Carbohydrates	5.0	2.5	1.25	0.63
Proteins	3.0	1.5	0.75	0.38
Iron	0.0001	0.00005	0.0	0.0
Calcium	0.2	0.1	0.05	0.03
Cholesterol	0.0025	0.00125	0.0	0.0
Total fat	0.33	0.165	0.08	0.04
Saturated fats	0.05	0.025	0.01	0.01
Sodium	0.5	0.25	0.13	0.06
Vit A	0.006	0.003	0.0	0.0
Vit B	0.00038	0.00019	0.0	0.0
Total solids (g)	9.09	4.54	2.27	1.14

for all five transfers, from 408.02 to 884.74 mg/L, similar to that attained at the first transfer for both 25% MRS plus 25% milk and 25% M17 plus 25% milk. The highest nisin concentration (3563.20 mg/L) before the fifth transfer was observed for MRS 25% plus skimmed milk 25%. A dilution of both media (MRS plus milk and M17 plus milk) provided levels of nisin activity (63.68 mg/L for 17.36% M17 plus 17.36% milk and 161.19 mg/L for 17.36% MRS plus 17.36% milk) five times lower than detected relatively to 25% concentration for the media assayed.

Jozala et al. *(28)* indicated that the preculture growth in MRS allowed nisin release by *L. lactis*; when compared with M17, nisin concentration was 1.7 times higher. Although nisin activity improved when milk was diluted with MRS and M17 broth to 25% of the original compounds. The nutrients and corresponding concentrations for three different media used in this work (Table 3), were shown to be correlated to the nisin activity data presented in Table 4. Nisin activity increased up to 97-fold from the first to the fifth transfer (90.14–8739.77 AU/mL) in milk diluted with water (4.54 $g_{\text{total solids}}$ at pH 6.8). Biomass (cells growth) increased up to threefold from the first to the fourth transfer (0.47–1.43 g/L) and in the fifth transfer decreased 1.5-fold (0.97g/L) (Fig. 1, Table 1).

Biomass (g_{DCW}/L) and nisin activity (AU/mL) in diluted skimmed milk (2.27 $g_{\text{total solids}}$ at pH 6.8) increased gradually through the transfers (Table 1). The levels of *L. lactis* biomass (0.49–1.78 g_{DCW}/L) and nisin activity (1255.16–20077.05 AU/mL) ranged from first to fifth transfer for each period of 36 h. However, in diluted skimmed milk with half total solids (1.14 $g_{\text{total solids}}$, pH 6.8), nisin activity reduced 11-fold from the first (273.21 AU/mL) to the fourth (25.89 AU/mL) transfers (Fig. 1, Table 1).

Comparing these results, the nisin activity through the transfers in the media with 2.27 $g_{\text{total solids}}$ was up to threefold and 85-fold higher than in those with 4.54 $g_{\text{total solids}}$ and 1.14 $g_{\text{total solids}}$, respectively (Fig. 1). From culture media 2.27 $g_{\text{total solids}}$ the maximum biomass (1.78 g_{DCW}/L)

Table 4
Nisin Production, Specific Production, Productivity, Proteins, and Sugar Consumption of *L. lactis* Growth, After 36 h, in Fermentation Processes to Diluted Skimmed Milk 2.27 $g_{total\ solids}$

	Parameters					Nisin production						
Samples	Time (h)	Temperature (°C)	pH	O_2 (%)	rpm	Halo (mm)	AU/mL	Log AU/mL	mg/L	Biomass g_{DCW}/L	Proteins g_{casein}/L	Sugars $g_{lactose}$/L
0	0	30	5.78	119.8	200	10.5	45.07	1.65	1.13	0.6	0.26	0.11
1	4	30	4.74	111.4	200	13.75	273.21	2.44	6.83	5.8	0.39	0.07
2	8	30	4.73	107.4	200	14.75	475.66	2.68	11.89	5.7	0.40	0.09
3	12	30	4.72	107	200	15.25	627.62	2.80	15.69	5.2	0.38	0.10
4	16	30	4.72	106.2	200	16.25	1092.70	3.04	27.32	6.5	0.40	0.20
5	20	30	4.72	104.5	200	16.5	1255.16	3.10	31.38	5.7	0.44	0.21
6	24	30	4.73	103.8	200	16.5	1255.16	3.10	31.38	6.4	0.44	0.21
7	28	30	4.73	103.3	200	16.5	1255.16	3.10	31.38	6.6	0.42	0.24
8	32	30	4.73	88.1	200	16.5	1255.16	3.10	31.38	5.9	0.28	0.23
9	36	30	4.74	88.5	200	18.25	3312.07	3.52	82.80	6.6	0.25	0.25

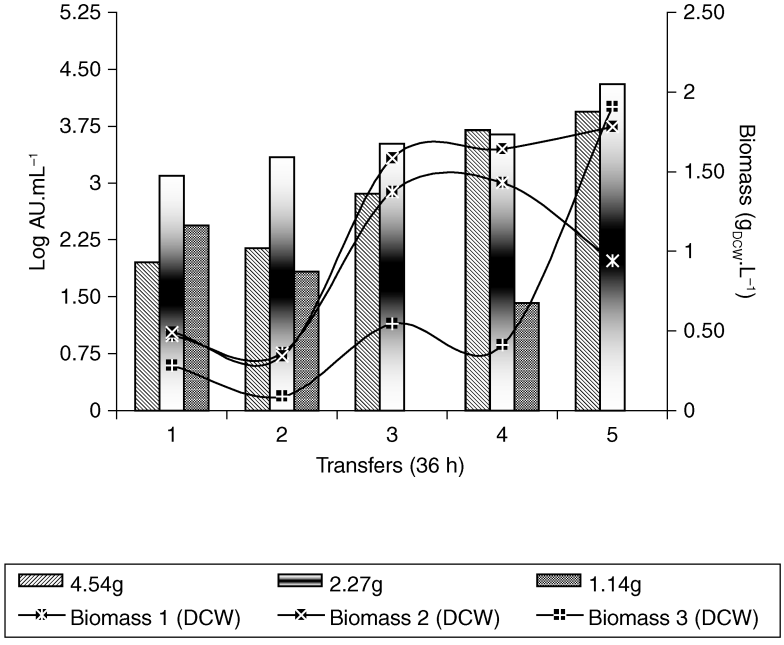

Fig. 1. Relation between nisin activity (log AU/mL) and biomass (gDCW/L) through the transfers in rotatory shaker assays into media with diluted skimmed milk at 4.54 $g_{total\ solids}$, 2.27 $g_{total\ solids}$, 1.14 $g_{total\ solids}$. AU/mL (log AU) and inhibition halo (H [mm]) was calculated through the equation: log [AU/mL = $10^{(0.2408 \times H - 0.8745)}$].

corresponded to the maximum nisin activity 20077.39 AU/mL = 501.93 mg/L, in the fifth transfer. However, in the same transfer from media with 4.54 $g_{total\ solids}$, the maximum biomass did not correspond to the maximum nisin activity 8799.77 AU/mL = 218.93 mg/L (Fig. 1, Table 1).

Milk Components to Formulate Artificial Media

Four different groups of assays were developed utilizing artificial compounds based on proportions of skimmed milk with 2.27 $g_{total\ solids}$, to evaluate which nutrient influenced more nisin activity (Table 5). In the assay made up of casein (0.75 g) and lactose (1.25 g), nisin activity was reduced 14-fold from the first to the second transfer (414.10–29.70 AU/mL) and increased up to twofold from the second to the fourth transfer (29.70–68.31AU/mL).

In the systems: (a) casein (0.75 g), lactose (1.25 g) plus calcium chloride (0.06 g); (b) casein (0.75 g), lactose (1.25 g) plus sodium citrate (0.01 g); and (c) casein (0.75 g), lactose (1.25 g), calcium chloride (0.06 g) plus sodium citrate (0.01 g), nisin activity was observed on the first and second transfers (Fig. 2, Table 2), the respective values were: (a) 475.66 and 45.07 AU/mL, (b) 68.31 and 45.07 AU/mL, and (c) 156.93 and 59.47 AU/mL.

The pH values on the assays with artificial compounds were high (ratio pH 6.5) and inhibited nisin activity. Release of intracellular nisin

Table 5
Artificial Media Compounds Based on Skimmed Milk 2.27 $g_{total\ solids}$

Nutrients	A	B	C	D
Carbohydrates	1.25	1.25	1.25	1.25
Proteins	0.75	0.75	0.75	0.75
Sodium	–	–	0.01	0.01
Calcium	–	0.06	–	0.06
Total solids (g)	2.0	2.06	2.01	2.07

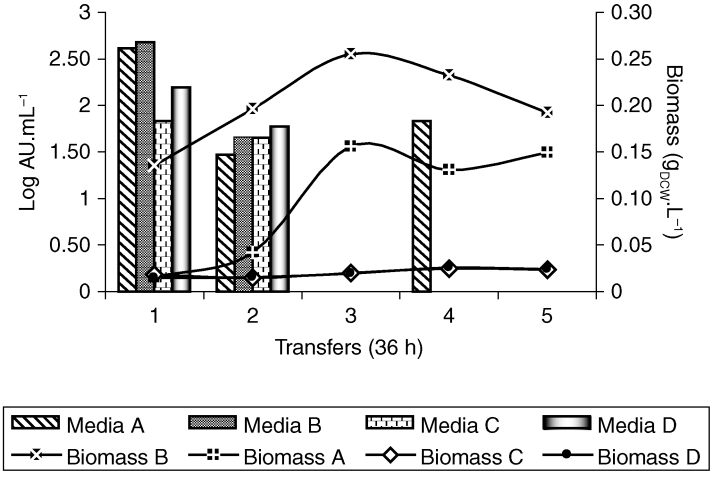

Fig. 2. Relation between nisin activity (log AU/mL) and biomass (g_{DCW}/L) through the transfers in rotatory shaker assays into media with artificial compounds media A; media B; media C; media D. AU/mL (log AU) and inhibition halo (H [mm]) was calculated through the equation: log [AU/mL = $10^{(0.2408 \times H - 0.8745)}$].

depends on the pH value of the growth media, in pH values lower than 6.0, the 80% expressed nisin is delivered to the media. On the other hand, when *L. lactis* grows in alkaline pH (pH > 6.0), most of the nisin is retained intracellular or within the cell membrane *(32–35).*

Cheigh et al. *(36)* observed the highest nisin activity early in the stationary phase (20 h, 30°C) 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 eightfold greater nisin activity than either M17 supplemented with 0.5% glucose or in MRS broth. The authors confirmed low levels of nisin activity in both MRS and M17 broth, although these media favored cellular growth, with similar results obtained in this study (10^7–10^9 CFU/mL). Chandrapati and O'Sullivan *(34)* observed a 50% increment in nisin activity using sucrose as the carbon source in M17 broth for *L. lactis* culturing, over two transfers. The authors observed that glucose was the optimal carbon source tested, with glycerol the least suitable. They also

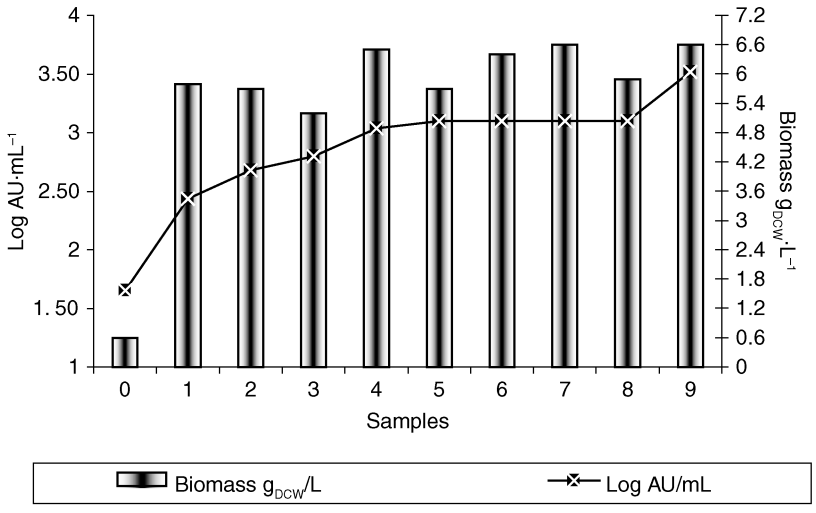

Fig. 3. Relation between nisin activity (log AU/mL) and biomass (g_{DCW}/L) of the samples in fermentor assay with skimmed milk 2.27 $g_{total\ solids}$ · AU/mL (log AU) and inhibition halo (H [mm]) was calculated through the equation: log [AU/mL = $10^{(0.2408 \times H - 0.8745)}$].

verified that the incorporation of either sodium or potassium phosphate into an artificial medium did not improve nisin production.

Single Batch Fermentation

In fermentation conditions, nisin activity was observed after 4 h (45.07 AU/mL) and stabilized in between 20 and 32 h process (1255.16 AU/mL). For the last 4 h fermentation (performing 36 h total process) the nisin production speeded up to threefold (3312.07 AU/mL). Oxygen demand was low in entire process and did not influence biomass or nisin production. The pH value was stabilized in 4 h cultivation (pH = 4.74 ± 0.2) and was maintained through the process. (Fig. 3, Table 4).

Flôres and Monte Alegre *(37)* investigated nisin activity during the fermentation batch with *L. lactis* ATCC 7962, nisin bactericidal effect was detected after 4 h fermentation when 40% biomass had been produced. Furthermore, the maximum nisin activity was in 9 h fermentation (pH 4.9); however it decreased in the following 24 h process. Previous work *(26)* utilized in single batch fermentation culture media with skimmed milk plus MRS broth and the maximal nisin activity ranged from 1376.97 AU/mL (8 h fermentation) to 5934.03 AU/mL (16 h fermentation).

In the present work, the composition of the diluted skimmed milk (2.27 $g_{total\ solids}$) with no extra supplementation was verified to be enough for *L. lactis* growth with concomitant nisin production. Nisin activity in diluted skimmed milk (2.27 $g_{total\ solids}$) with no supplementation was similar to the activity of nisin expressed in the mixture of 25% skimmed milk

with 25% MRS broth in the previous work *(27)*. Liu et al. *(38)* observed a specific nisin formation of 5.4×10^6 AU/g at pH 5.5 in M17 broth added with lactose as a carbon source, for immobilized *L. lactis* in continuous fermentation, where nisin formation was reported to be greatly influenced by medium dilution rate.

Conclusions

The culture media made up of diluted skimmed milk (2.27 $g_{\text{total solids}}$) was shown to support better conditions for nisin production and activity by *L. lactis*. The mechanism for this improvement is unclear. Quality of a natural product cannot be reproduced in an artificial way, because milk composition is an extremely complex group of the natural nutrients. In this work *L. lactis* cells developed in minimum concentration of diluted skimmed milk; however, artificial compounded media did not favor cell adaptation and, consequently, failed nisin production.

Besides that skimmed milk dilution for fermentation batch and nisin by *L. lactis* cells showed similar behavior when compared with fermentation with skimmed milk plus MRS broth *(26)*. This research shows the utilization of a low-cost growth media (diluted skimmed milk) to antimicrobial production with wide applications. Furthermore, the utilization of milk subproducts can be exploited (milk whey), because milk whey contains considerable levels of casein and lactose and these nutrients are observed to improve nisin production. In Brazil, 50% of milk whey is disposed with no treatment in rivers and because of high-organic matter concentrations milk whey is considered an important pollutant. In this particular case, an optimized production of an antimicrobial would be lined up with industrial disposal recycling.

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