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Full Length Research Paper

Increase of nisin production by *Lactococcus lactis* in different media

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Nisin production related to the growth conditions of *Lactococcus lactis subsp. lactis* ATCC 11454, the effects of various media components and concomitant release of nisin into the media, were studied through transfers (five times). Nisin production was assayed by agar diffusion using *Lactobacillus sake* ATCC 15521 as the sensitive test organism. The expression of nisin was strongly influenced by the addition of skimmed milk to both MRS and M17 broth, with the highest production obtained after the second and the fifth transfers, respectively, with maximum expression after 36 h of incubation.

Key words: Nisin, *Lactococcus lactis*, *Lactobacillus sake,* bacteriocin, milk.

INTRODUCTION

Nisin is a naturally occurring antimicrobial peptide and was discovered in 1928 (Hurst, 1967; Montville and Chen, 1998). Nisin has been applied in food preservation (Delves-Broughton et al., 1990; Thomas and Delves-Broughton, 2001) and dental care products (Turner et al., 2004).

Nisin inhibits spore germination and growth of gram-positive bacteria and for this reason, it is widely used as a natural preservative (Vessoni Penna and Moraes, 2002a; Thomas et. al., 2002). Previous reports have described interactions between nisin with EDTA that resulted in enhanced antimicrobial effect against Gram-negative bacteria (Gill and Holley, 2003)

Nisin solubility and stability increases substantially with increasing acidity. Nisin is stable at pH 2 and can be autoclaved at 121ºC. Under alkaline pH there is an increasing loss of activity, with complete inactivation after 30 min at 63ºC at pH 11 (Hansen et al., 1991).

Relating nisin expression to growth conditions of *Lactococcus lactis subsp. lactis* ATCC11454, in this study, the effects of various media components through five transfers of cells to fresh media and, concomitant release of nisin into the media, were evaluated.

MATERIALS AND METHODS

The nisin-producing *L. lactis* ATCC 11454 and a nisin-sensitive indicator species, *Lactobacillus sake* ATCC 9221, were used in this study. Stock cultures were maintained at -80°C in MRS broth (Man Rugosa Shepeer-Bacto Lactobacilli, DIFCO) with 40% (v/v) glycerol.

The nisin standard was Nisaplin® (Sigma Chemical) prepared with a solution of 0.02 M HCl. Correlating several concentrations of a nisin standard (10^0 to 10^5 AU mL^{-1}) with the diameter of the inhibition halo (H, mm), the activity of nisin from sample cultures was determined and expressed in arbitrary units per mL (10^0 to 10^5 AU/mL). Based on the calibration curves between AU mL^{-1} and IU

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Table 1. Nisin activity, productivity and specific production for every transfer, each incubated at 100 rpm/30°C/36 h, with *Lactococcus lactis* ATCC 11454 cultures transferred to fresh media with either synthetic MRS or M17 components.

Media		pH	Transfer	Activity (AU/mL)	Nisin (mg/L)	Specific production (mg/DCWg)	Productivity (mg/DCWg .h^{-1})
Composition	Pre Inoculum						
		6.15	1	12.69	0.317	0.58	0.02
		6.15	2	12.99	0.325	0.45	0.01
100% MRS	MRS	5.65	3	11.57	0.289	0.44	0.01
(pH=6.5)		5.33	4	12.99	0.325	0.52	0.01
		5.12	5	21.06	0.527	2.49	0.08
		7.1	1	6.98	0.18	0.01	0.01
		7.3	2	6.98	0.18	0.01	0.01
100%M17	M17	6.8	3	6.98	0.18	0.01	0.01
(pH=6.9)		7.0	4	6.98	0.18	0.01	0.01
		7.2	5	6.98	0.18	0.01	0.01
		5.9	1	6.98	0.18	0.01	0.01
50%MRS +		4.7	2	6.98	0.18	0.01	0.01
0.13% suppl.	MRS	4.9	3	6.98	0.18	0.01	0.01
(pH=6.13)		4.7	4	1516.44	37.91	2.71	1.05
		4.8	5	9242.72	231.07	17.30	6.42
		6.0	1	6.98	0.18	0.01	0.01
50%M17 +		6.0	2	6.98	0.18	0.01	0.01
0.13% suppl.	M17	6.1	3	6.98	0.18	0.01	0.01
(pH=6.28)		6.1	4	7472.23	186.81	13.37	5.19
		6.3	5	6.98	0.18	0.01	0.01
		5.1	1	8.78	0.62	0.01	0.000011
100%MRS +		5.2	2	11.57	1.96	0.01	0.000014
0.5% sucrose.	MRS	5.2	3	17.12	9.75	0.02	0.000019
(pH=6.75)		4.9	4	23.09	34.87	0.02	0.000024
		5.1	5	12.99	3.16	0.02	0.000017
		5.5	1	647.78	16.19	1.22	0.45
50%M17 +		5.6	2	1102.30	27.56	1.77	0.77
0.14% sucrose	M17	5.7	3	801.26	20.03	1.49	0.56
(pH=6.94)		5.9	4	7472.23	186.81	13.37	5.19
		6.0	5	6040.88	151.02	11.31	4.19
		4.5	1	6.98	0.18	0.01	0.01
100%M17 +		4.5	2	6.98	0.18	0.01	0.01
12.5% sucrose	M17	4.5	3	223.71	5.59	0.42	0.16
(pH=6.27)		4.5	4	223.71	5.59	0.40	0.16
		4.4	5	11432.73	285.82	21.40	7.94

mL^{-1}, 1.09±0.17 AU corresponding to 1.0 IU (40 IU = 1 µg of pure nisin A). A standard solution of nisin, utilized in all the assays for calibration of the assay, showed 0.0250 mg of nisin corresponding to 10^3 AU/mL.

For nisin activity detection, the cell suspension was centrifuged at 12,000xg 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^{-1} of medium) by the agar diffusion assay 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% of bacteriologic 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.

RESULTS AND DISCUSSION

Although MRS and M17 media have been reported as suitable media for promoting growth and nisin expression with *L. lactis* (Cheig et al., 2002), the recommended concentrations for the synthetic media did not promote the release of nisin by the bacteria (Table 1). However, at half the recommended concentrations, 50% MRS plus

Table 2. Nisin activity, productivity and specific production for every transfer, each incubated at 100 rpm/30°C/36 h, with *Lactococcus lactis* ATCC 11454 cultures transferred to fresh media containing milk.

Media		pH	Transfer	Activity (AU/mL)	Nisin (mg/L)	Specific production (mg/DCWg)	Productivity (mg/DCWg .h^{-1})
Composition	**Pre inoculum**						
100% Milk 1 (ph=6.80)	MRS	5.1	1	4052.52	101.31	2.81	7.61
		4.2	2	16321.12	408.03	11.33	26.18
		4.3	3	35389.55	884.74	24.58	65.71
		4.7	4	22243.34	556.08	15.45	39.81
		4.8	5	16321.12	408.03	11.33	30.55
100% Milk 2 (pH=6.82)	M17	5.5	1	26.07	0.65	0.02	0.10
		4.8	2	4052.52	101.31	2.81	11.39
		4.9	3	7527.05	188.18	5.23	16.33
		4.9	4	25967.19	649.18	18.03	48.61
		4.7	5	19053.51	476.34	13.23	26.95
25%M17 +25%milk (pH=6.17)	M17	6.2	1	16321.12	408.03	11.33	30.63
		4.8	2	65731.72	1643.29	45.65	105.42
		4.7	3	65731.72	1643.29	45.65	122.05
		5.0	4	41314.26	1032.86	28.69	73.94
		4.8	5	142527.94	3563.20	98.98	266.79
25%MRS +25%milk (pH=6.12)	MRS	4.7	1	35389.55	884.74	24.58	66.42
		4.6	2	142527.94	3563.20	98.98	400.72
		4.7	3	35389.55	884.74	24.58	76.80
		4.7	4	89582.88	2239.57	62.21	167.68
		4.8	5	35389.55	884.74	24.58	50.05
17.36%MRS + 17.36%milk + 0.13% suppl. (pH= 6.35)	MRS	4.5	1	26.07	0.65	0.02	0.05
		6.0	2	26.07	0.65	0.02	0.04
		5.4	3	26.07	0.65	0.02	0.05
		6.3	4	6447.63	161.19	4.48	11.54
		5.4	5	6447.63	161.19	4.48	12.07
17.36%M17+ 17.36%milk + 0.13% suppl. (pH= 6.76)	M17	6.5	1	26.07	0.65	0.02	0.05
		5.1	2	26.07	0.65	0.02	0.04
		5.1	3	26.07	0.65	0.02	0.05
		5.7	4	861.94	21.55	0.60	1.54
		5.6.	5	2547.12	63.68	1.77	4.77
17.86%M17+ 17.86%milk + 0.14% sucrose (pH= 6.74)	M17	5.7	1	26.07	0.65	0.02	0.05
		5.1	2	26.07	0.65	0.02	0.04
		5.1	3	26.07	0.65	0.02	0.05
		5.3	4	8787.19	219.68	6.10	15.73
		5.3	5	7527.05	188.18	5.23	14.09

Arbitrary Unity/ml (AU/ml)= $10^{(0.2689^{*}\ x\ +\ 1.3893)}$, where x= the diameter of the halo (H, mm)
Nisin concentration (mg/L)= (x) * 0.025, where x= Activity (AU/ml), and 0.025 the conversion factor of the standard Nisin solution (0.025mg/ml =10^3 AU/ml).
Productivity (mg/L h-1) = (x)/36, where x = Nisin concentration (mg/L).
Specific production: mg/g h-1 = (x)/DCW, where x = Productivity (mg/ L h-1), and DCW = dry cell weight(g/L).

0.13% supplements, 50% M17 plus 0.13% supplements and 50% M17 plus 0.14% sucrose, provided equivalent nisin activity between the fourth and fifth transfer cultures.

The sucrose added to MRS or M17 in previous work, favors the production of nisin by cells (Vessoni Penna and Moraes, 2002b). The addition of 0.14% sucrose into 50% M17 increased nisin expression at the fourth transfer, while the addition of 12.5% sucrose into 100% M17 stimulated nisin production at the fifth transfer. The release of the nisin by cells is dependent on the media pH value.

Cheigh et al. (2002) 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 8-fold greater nisin expression than either M17 supplemented with 0.5% glucose or in MRS broth. The authors confirmed low levels of nisin expression in both MRS and M17 broth, although these media favored cellular growth, with similar results obtained in this study (10^7 to 10^9 CFU/mL). Chandrapati and O'Sullivan (1998) observed a 50% increment in nisin expression 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 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 media concentrations to 17.36% milk added with 17.36% MRS or 17.36% M17, reduced nisin activity sixteen times, from the maximum of 3563.20 mg/L obtained of the 25% M17 (MRS + 25% milk), to 63.68 mg/L obtained of the 17.86% M17 (MRS + 17.86% milk), related to the fifth transfer (Table 2). In these formulations, in the first, second and third transfers, the detection of an inhibition zone was negligible but significant nisin production could be observed from the fourth transfer culture.

In the formulation using MRS + 17.86% milk, nisin production values were similar in the fourth and fifth transfers, 161.19 mg/L. The highest productivity value, 12.07 mg/L/h, was detected in the fifth transfer culture. In the formulation using M17 + 17.86% milk, nisin production at the fourth transfer was 21.55 mg L^{-1} and 63.68 mg L^{-1} in the fifth transfer culture. In media consisting of M17 + 17.86% milk + 0.14% sucrose, nisin production in the fourth transfer culture was 219.68 mg/L and 188.18 mg/L in the fifth transfer. These results show the influence of sucrose in M17 broth on the release and detection of nisin, even though the original concentrations of the basic media components were 60% of manufacturer's recommended formulation (Table 2)

The type of components and their concentration in 100% milk media (milk 1 and milk 2), at pH 4.7, favored the expression of the nisin (Table 2). In these results the pre-inoculum growth in MRS favored the release of nisin by *L. lactis;* there was 1.7 times greater concentration of nisin relative to the pre-inoculum grown in M17.

Nisin expression was four times higher for the second and third transfer cultures in milk 1 relative to milk 2, with the pre inoculum derived from cells grown in MRS for 100% milk 1 and, in M17 for 100% milk 2. These results emphasize the importance of the type of medium used for the pre-inoculum culturing of *L. lactis*.

The formulations of 25% milk plus 25% M17 and 25% milk plus 25% MRS were found to stimulate optimal bacteriocin production. Both media define the final composition of the nutrients favorable to nisin expression and release into media at a final pH between 4.6 and 4.8. (Table 2).

The 25% milk concentration showed a positive influence in the formation and release of nisin by the cells and was shown to be the best component to add to 25% M17 or 25% MRS. Nisin production increased consistently from first to fifth transfer in the M17+milk media; this formulation was selected for future studies.

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