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Full Length Research Paper

Effect of non-nutritional factors on nisin production

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When attempting to improve production of nisin, understanding the effect of non-nutritional factors is essential owing to a lack of adequate information about these factors among various investigations. In order to assess some of non-nutritional factors and how they influence the nisin production in batch cultivation, a laboratory scale study was performed. *Lactococcus lactis* subsp. *lactis* ATCC 11454 produced nisin and *Micrococcus luteus* ATCC 10240 was used in bioassay measurement as the nisin-sensitive strain. The age and size of inoculum, initial pH value of the medium and flask volume/medium volume (F/M) ratio, temperature as well as agitation were studied by changing one factor at a time while keeping others constant in de Man, Rogosa and Sharpe (MRS) medium. Our results implied that pH value was positively related to increase nisin production. Two other important factors for a maximum nisin production were found to be agitation and flask volume/medium volume (F/M) ratio. Inoculum size more than 2.5% (v/v) had no effect on nisin production. The most suitable condition for inoculum age was 32-hour-old culture (at the end of log phase) and 27°C temperature provided maximum nisin production.

Key words: Nisin, *Lactococcus lactis*, temperature, bioassay, batch fermentation.

INTRODUCTION

Nisin, a bacteriocin, is ribosomally synthesized and used alone or in combination with other preservation technologies as a safe preservative (Devlieghere et al., 2004; Cleveland et al., 2001). Nisin is produced by some strains of *Lactococcus lactis* and is active against some Gram-positive bacteria, including pathogenic and food spoilage microorganisms such as clostridia, bacilli, *Staphylococcus aureus*, micrococci, lactobacilli and *Listeria monocytogenes*. Nisin is used in over 48 countries, has the Food and Drug Administration (FDA) approval (Liu et al., 2005a; Deegan et al., 2006) and is authorized for food preservation in the European Union by Directive 95/2/EC on food additives (Anton et al., 2006). In spite of achievements in the understanding of microbial physiology and molecular biology, cultivation optimization remains largely a crucial process. A program for cultivation improvement may begin by measuring product yield as a response to nutritional (carbon, nitrogen, phosphorus sources) and non-nutritional (temperature, agitation, inoculum age,

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Abbreviations: F/M, Flask volume/medium volume; **MRS,** de Man, Rogosa and Sharpe medium; **FDA,** food and drug administration; **BHI,** brain heart infusion broth; **CFU,** colony-forming units; **RP-HPLC,** reversed-phase high performance liquid chromatography.

Table 1. Fermentation conditions of non-nutritional factors.

Fermentation conditions						
Factors	**Temperature (°C)**	**Agitation (rpm)**	**Inoculum age (h)**	**Inoculum size (%v/v)**	**pH**	**Flask volume/medium volume ratio [a]**
Temperature	Variable [b]	100	32	1.0	6.50	2.50
Agitation	30	Variable	32	1.0	6.50	2.50
Inoculum age	30	100	Variable	1.0	6.50	2.50
Inoculum size	30	100	32	Variable	6.50	2.50
pH	30	100	32	1.0	Variable	2.50
Flask volume/medium volume ratio	30	100	32	1.0	6.50	Variable

[a]Different volumes of culture medium were used in various vessels (Erlenmeyer flasks and bench-scale bioreactors) for evaluation of nisin production. Accordingly, by varying the flask volume/medium volume (F/M) ratio (Vázquez et al., 2004) different culture medium volumes in 250 ml Erlenmeyer flasks were studied. For example, F/M ratio of 10 represents a 250 ml Erlenmeyer flask contained 25 ml of culture medium (MRS).
[b]The levels of each factor is seen in Table 2.

inoculum size, pH) factors (Strobel and Sulivan, 1999). Non-nutritional factors strongly affect on yield and evaluating their effects and optimization, can improve fermentation process. This leads to maximum production without any changes in components concentration or the need for adding supplements to the culture medium.

Despite several investigations on the effect of nutritional factors on nisin production in laboratory scale (Kim et al., 1997; Guerra and Pastrana, 2001; Penna and Moraes, 2002; Penna et al., 2005; Liu et al., 2005b; Lv et al., 2005; Jozala et al., 2007), any standard non-nutritional conditions have not been reported. Except for temperature, the non-nutritional conditions (such as Inoculum age, inoculum size, initial pH value, flask volume/medium volume (F/M) ratio and agitation) which have used by investigators are not the same and in some cases are very different. Whereas non-nutritional factors variations cause different results, it fails to realize that these changes are related to media components or non-nutritional factors variations. In this study, the influence of the most important non-nutritional factors on the production of nisin was studied in order to obtain the optimum fermentation conditions in standard medium.

MATERIALS AND METHODS

Bacterial strains and media

Lactococcus lactis subsp. *lactis* ATCC 11454 as the nisin producer and *Micrococcus luteus* ATCC 10240 as the nisin-sensitive strain were kindly provided by the Persian Type Culture Collection (PTCC). All stock cultures were maintained at –80°C and in 20% glycerol. *L. lactis* subsp. *lactis* and *M. luteus* were grown in de Man, Rogosa and Sharpe (MRS) broth (HiMedia Laboratory, India) and brain heart infusion (BHI) broth (HiMedia Laboratory, India), respectively. The working cultures were maintained on agar slants of the same media at 4°C.

Nisin bioassay

Nisin bioassay was determined by agar diffusion method (Pongtharangkul and Demirci, 2004). In BHI medium, 0.75% agar (Bacto agar, Difco) and 1% (v/v) Tween 20 (Sigma Chemical Co., St. Louis, Mo.) were added and the medium was boiled and then sterilized. After temperature adjustment to 40°C, the medium was inoculated with 1% v/v of a 24-h culture of *M. luteus* which was incubated at 37°C with an optical cell density of 1.7 at 600 nm which gave approximately 10^8 colony-forming units (CFU) of the microorganism per ml of the agar medium. Sterile Petri dishes (100 × 15 mm) were filled with 25 ml of the inoculated bioassay medium and after solidification of the agar medium, by using a sterilized stainless steel borer (7-mm outer and 5-mm inner diameter), four holes were bored on each plate. Then, 50 µl of each nisin sample was placed into each well in triplicate and the fourth well was filled with blank (50 µl of 0.02 M HCl). The plates were incubated at 4°C for 24 h (pre-diffusion of nisin) and then incubated at 37°C for another 24 h. The plates were examined for diameter of inhibition zones using a digital caliper (AACO, China) to the nearest 0.01 mm and the results of three measurements were averaged.

A stock solution of nisin (1000 IU/ml) was prepared by dissolving 0.025 g of commercial nisin 10^6 IU/g (Sigma Chemical Co., St. Louis, Mo.) in 25 ml of sterile diluent solution of 0.02 M HCl, in order to construct standard curve. Standard nisin solutions at the range of 500, 400, 300, 200, 100, 50, 25, 10, 5 and 0 IU/ml were prepared by using 0.02 M HCl. The standard curve was computed using the diameters of inhibition zones against the logarithm_{10} of nisin concentrations.

Fermentation conditions and levels of non-nutritional factors

Erlenmeyer flasks (250 ml) containing MRS broth were inoculated, using *L. lactis* culture. Samples were withdrawn from the flasks during incubation period every 8 h. In this study, according to our experimental design, one factor changed while all others were held constant in each experiment. All experiments were performed in triplicate. The full fermentation conditions of non-nutritional factors are shown in Table 1.

As mentioned earlier, various levels of non-nutritional factors were used by researchers and for this reason a wide range of non-

Table 2. Factors and levels description.

Factors	Level 1	Level 2	Level 3	Level 4	Level 5
Temperature (°C)	24	27	30	33	36
Agitation (rpm)	0	50	100	150	200
Inoculum age (h)	8	16	24	32	40
Inoculum size (%v/v)	1.0	2.5	5.0	7.5	10.0
pH [a]	4.50	5.50	6.50	7.50	-
F/M ratio	10.00	5.00	3.33	2.50	-

[a] The pH of culture medium was adjusted to appropriate pH with NaOH (4 M) or concentrated hydrochloric acid (Merck, Germany). The pH of MRS broth is 6.50.

nutritional levels were selected for evaluation of their effects on the nisin production. In Table 2, the level of each factor is seen.

Sample preparation

A sample of fermentation broth was immediately adjusted to pH 3.0 using concentrated HCl (Merck, Germany) and 0.1% v/v of Tween 20 (Sigma Chemical Co., St. Louis, Mo.), in order to avoid any non-specific adsorption of nisin onto the container's surfaces. After heating the sample at 90°C for 5 min and centrifugation at 12,000 x g for 10 min at 4°C, the supernatant was collected and filtered through a 0.22 µm membrane filter (Millipore® Corp., Bedford, MA) and stored frozen at -20°C for later use (Jozala et al., 2005; Pongtharangkul, 2006). The quantification of nisin was performed by agar well diffusion bioassay method.

Inoculum age standardization

To ensure that equal number of the nisin producer strain cells were inoculated into the culture media each time and also for evaluation of inoculum age effect, a specific OD_{600nm} using a spectrophotometer (Spectronic 1201, Milton Roy Co., Rochester, NY) was used. At the same time, for determination of bacterial cells numbers, viable cell counts using MRS agar (HiMedia Laboratory, India) were performed by the serial dilution method in potassium phosphate buffer solution (Merck, Germany) with pH adjusted to 7.00. After incubation for 72 h at 30°C, the colonies were counted (Bunthof et al., 1999). Colony count was carried out in triplicate for each inoculum age. Each inoculum age was prepared in a 250 ml Erlenmeyer flask containing 75 ml MRS broth with pH 6.50 and shaked at 100 rpm and 30°C.

Nisin purification and HPLC analysis

After a 400 ml culture of *L. lactis* was grown for 32 h at 100 rpm and 30°C, cells were removed by centrifugation (10,000 x g, 30 min, 4°C). Culture supernatant were made up to 30% saturation by slow addition of solid ammonium sulfate (Merck, Germany) and kept overnight at 4°C with gentle stirring. Precipitated protein was removed by centrifugation (12,000 x g, 30 min, 4°C) and resuspended in 2 ml sterile solution of 0.02 M HCl (Merck, Germany) and dialyzed against 4 l of the same solution for 24 h in Spectra/Por® no. 7 dialysis tubing (Spectrum laboratories Inc., USA, molecular weight cut off, 2000 Daltons) with two changes of the solution (Daoudi et al., 2001). This fraction was concentrated by freeze-drying, resuspended in 1.5 ml of 0.02 M HCl sterile solution and filtered through a 0.22 µm membrane filter (Millipore® Corp., Bedford, MA). The preparation and a 2% standard nisin (Sigma Chemical Co., St. Louis, Mo.) solution (which was prepared by suspending nisin powder in sterile solution of 0.02 M HCl) were further purified by gel filtration chromatography on Sephadex G-25 as described by Pirad et al. (1992). After gel filtration chromatography, fractions containing nisin (determined by bioassay method) were polled and dialyzed against 4 l of 0.02 M HCl with two changes of the solution and concentrated by freeze-drying. The lyophilized samples were injected into a Knauer HPLC unit (Model K-1001, Knauer, Germany) equipped with an analytical reversed-phase (RP) C18 column (Eurosil Bioselect, Knauer, Germany) for retention time measurement (Chollet et al., 2008). Peak with retention time from 46.0 to 47.0 min was considered as nisin and identified by comparing retention time with that of standard. Both standard nisin and sample nisin (purified from the culture medium) collected eluents with retention time from 46.0 to 47.0 min were assayed for activity against *M. luteus* ATCC 10240 for biological activity confirmation.

RESULTS

The growth of *Lactococcus lactis*

The results of colony count and cell density measurement at appropriate time are summarized in Figure 1. It is shown that the cell density at 600 nm was increased after 16 h (from 0.2 to 1.5) and was reached to its maximum level in 32 h (1.9). After this time, cell density remained constant. Cell count was also increased after 16 h and the maximum level was observed in 32 h ($1.8 \pm 0.4 \times 10^9$ CFU/ml). Cell count was decreased 40 h after starting of incubation time.

The effect of non-nutritional factors on nisin production

Initial pH value of the medium

The pH effect on the production of nisin is shown in Figure 2. When MRS broth in different initial pH values were assayed, nisin production was strongly influenced by the initial pH values of MRS medium. Maximum nisin production (with a titer of 465 IU/ml after 32 h) was

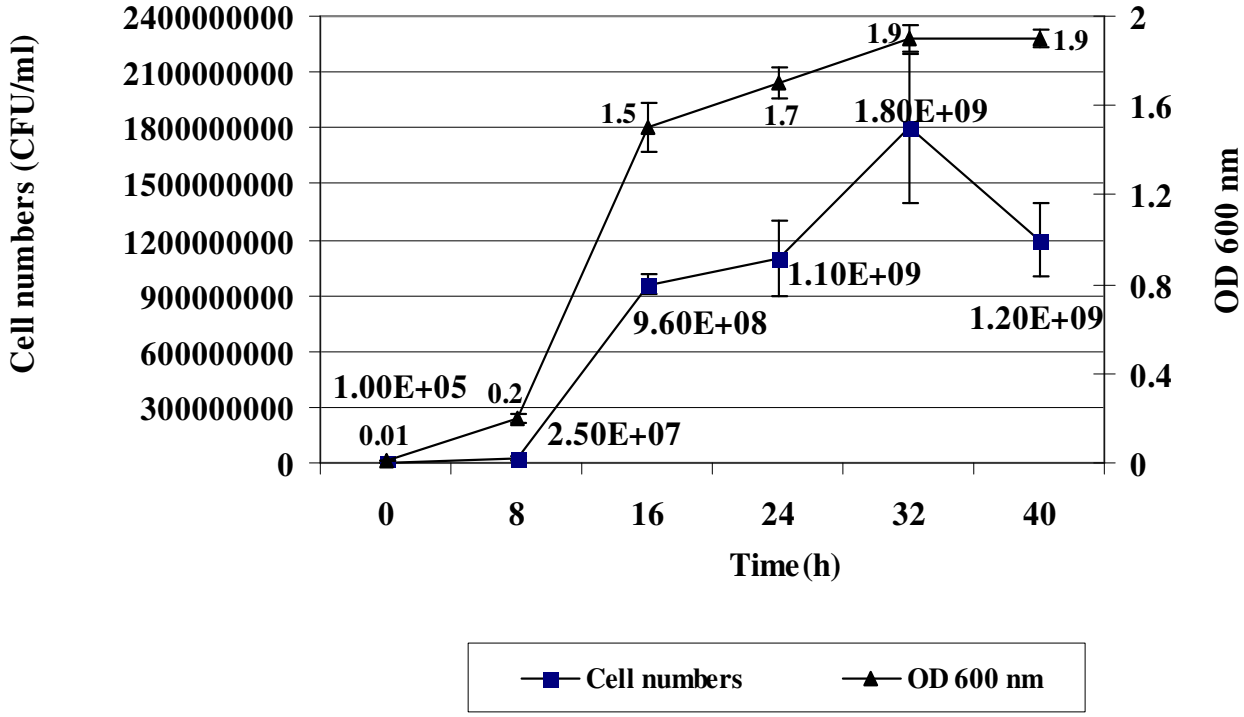

Figure 1. Cell numbers and optical density of each inoculum age at 600 nm.

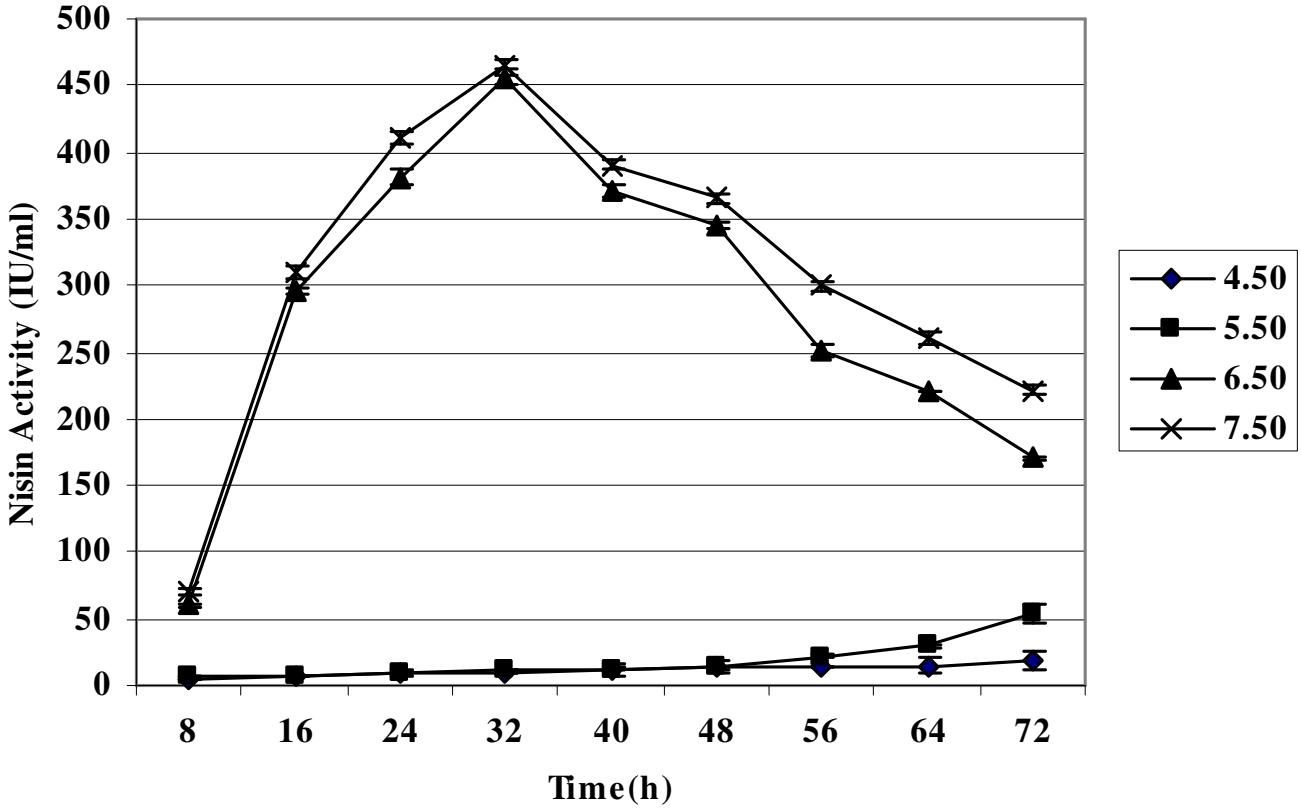

Figure 2. The effect of initial pH value of the medium on nisin production.

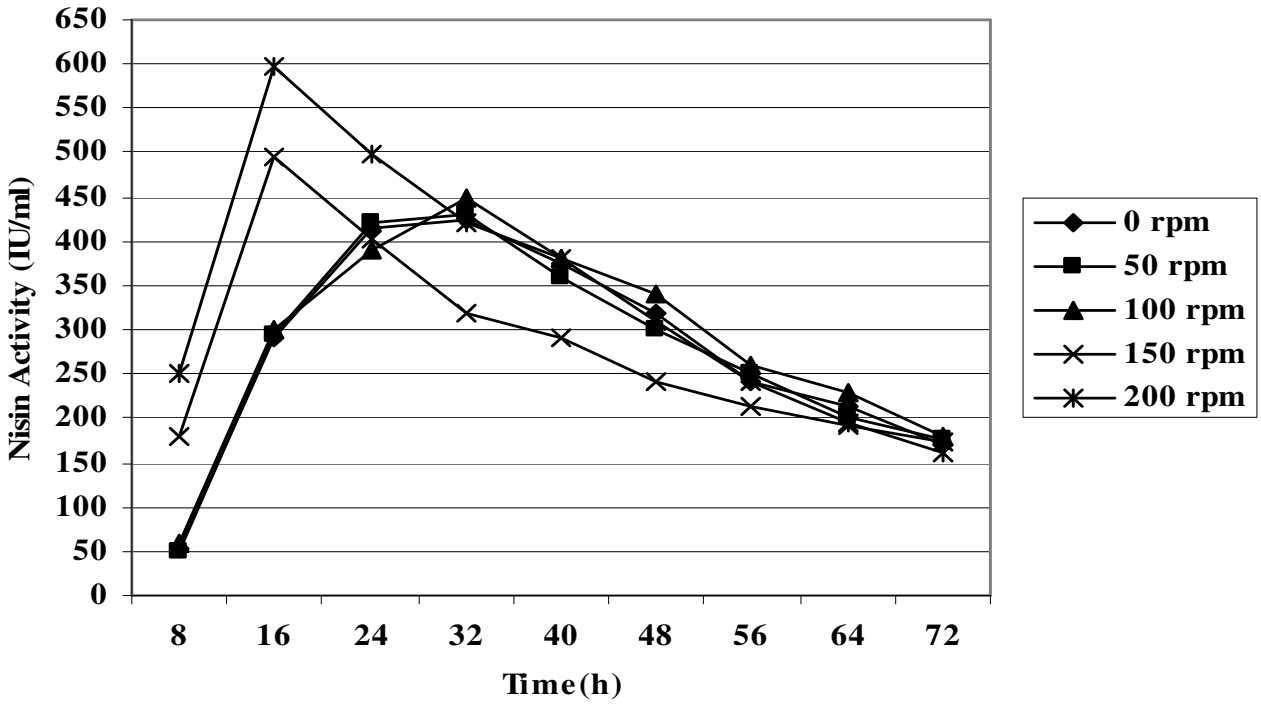

Figure 3. Effect of agitation on nisin production.

observed at pH 7.50.

Agitation

Figure 3 indicates the effect of agitation on the production of nisin in MRS broth. Maximum nisin production was observed after 16 h at 200 rpm (598 IU/ml). Agitations between 0 and 100 rpm had a similar pattern on nisin production but the maximum production time was shorter at 150 rpm as well as 200 rpm (after 16 h).

F/M ratio

The influence of F/M ratio (flask volume/medium volume ratio) on the nisin production in MRS medium is shown in Figure 4. The 10 F/M ratio was associated with maximum nisin production (649 IU/ml) after 24 h. Nisin production was decreased with F/M ratio reduction to 2.50 and maximum nisin production time was shifted from 24 h to 32 h.

Inoculum size

Nisin production was increased by increasing the inoculum size and maximum nisin titer was achieved after 24 h at 7.5% v/v with 588 IU/ml (Figure 5). There was no important influence between 2.5 and 10% v/v inoculum sizes on the maximum production of nisin (560, 580, 588 and 565 IU/ml for 2.5, 5.0, 7.5 and 10.0% inoculum sizes, respectively) but higher inoculum sizes (7.5 and 10.0%) reduced maximum nisin production time from 32 to 24 h.

Inoculum age

The influence of inoculum age on the production of nisin is shown in Figure 6. Maximum nisin production with 1% v/v of 32-h-old inoculum age was observed after 32 h (470 IU/ml). Nisin production was influenced comparably by 16, 24 and 40-h-old inoculum ages and 8-h-old inoculum age had clearly the lowest effect and 32-h-old was the best inoculum age for nisin production.

Temperature

The effect of incubation temperature on nisin production in MRS broth is shown in Figure 7. Maximum nisin production was observed at 27°C after 32 h (482 IU/ml). Nisin production was nearly equal between 27 and 33°C (482, 450, 440 IU/ml in the 27, 30°C conformably) and it can be disregarded at 36°C. A decrease in the maximum nisin production time was seen when temperature was

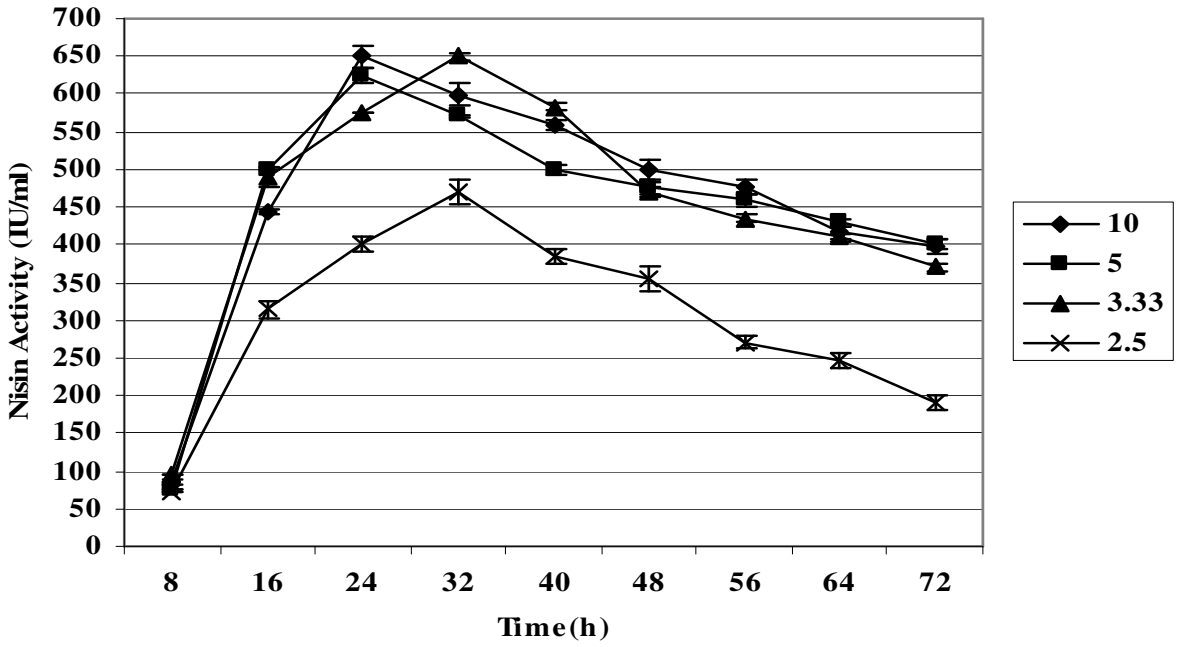

Figure 4. Effect of F/M ratio on nisin production.

reduced to 24°C.

RP-HPLC analysis

As can be seen in Figure 8, the RP-HPLC chromatographic profile were quite similar and showed the peaks with retention times from 46.0 to 47.0. According to results obtained with bioassay method (data not shown), the column eluents with retention time from 46.0 to 47.0 min had the same nisin bioactivity.

DISCUSSION

Nisin is the only purified bacteriocin that has received acceptance in countries worldwide as a biopreservative. Preliminary experiments are usually done in shaken flasks or similar culture vessels to minimize time and cost involved in the study of nutritional and non-nutritional factors involved in cultivation improvement. Optimal nisin production usually needs a suitable complex medium (nutritional effect) and well-controlled parameters of non-nutritional factors such as pH, temperature, agitation and aeration (Arauz et al., 2009). The influence of nutritional factors on the production of nisin have been reported (Kim et al., 1997; Guerra and Pastrana, 2001; Penna and Moraes, 2002; Penna et al., 2005; Liu et al., 2005b; Lv et al., 2005; Jozala et al., 2005; Jozala et al., 2007). In these studies, various fermentation conditions were used by researchers for evaluation of nisin production using *L. lactis* subsp. *lactis* ATCC 11454 as nisin producer strain and except for temperature, the other non-nutritional factors used for evaluation of nisin production were highly variable and a very low similarity was seen among them in this regard. Our study evaluated the effect of the most important non-nutritional factors on nisin production. We tried to find out the range of optimal fermentation conditions for nisin production in a standard culture medium (MRS) without any changes in components concentration of this medium or adding supplements into it.

Our study showed that production of nisin in acidic pH values (4.50 and 5.50) is negligible. It means that the initial pH value is a critical factor in nisin production (Figure 2). However, maximum stability and solubility of nisin is reported in acidic pH values (Laridi et al., 2003), but production of nisin in these conditions is very low. The optimum initial pH values for maximum nisin production were found at 6.50 and 7.50 which is confirmed by Jozala et al. (2005) study. They found that optimum initial pH value for nisin production is between 6.12 and 6.94 but according to our study, 7.50 initial pH value was also suitable for production of nisin.

Agitation is important for adequate mixing, mass transfer and heat transfer. It not only assists mass transfer, but also maintains homogeneous chemical and physical conditions in the culture by continuous mixing. On the other hand, agitation and aeration may affect the fermentation process because of changes in dissolved O_2 and CO_2 (Roukas, 2006). In this regard and according to our experience, F/M ratio (Vázquez et al., 2004) and agitation are the two factors which affect on nisin production in

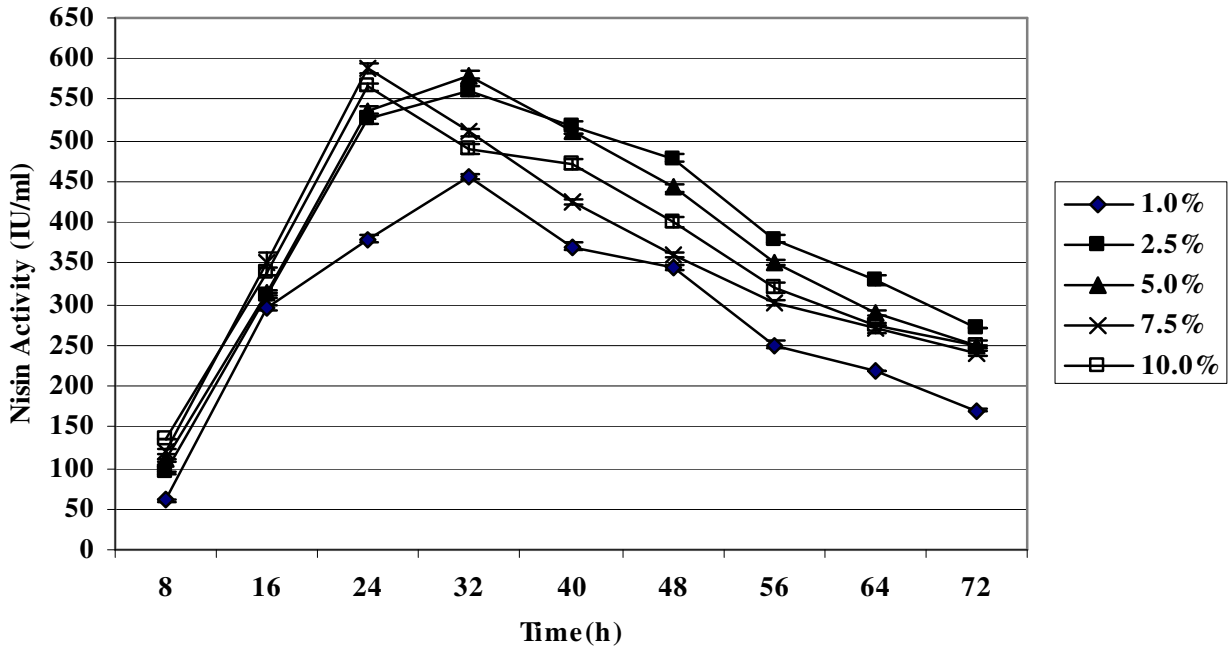

Figure 5. Effect of inoculum size on nisin production.

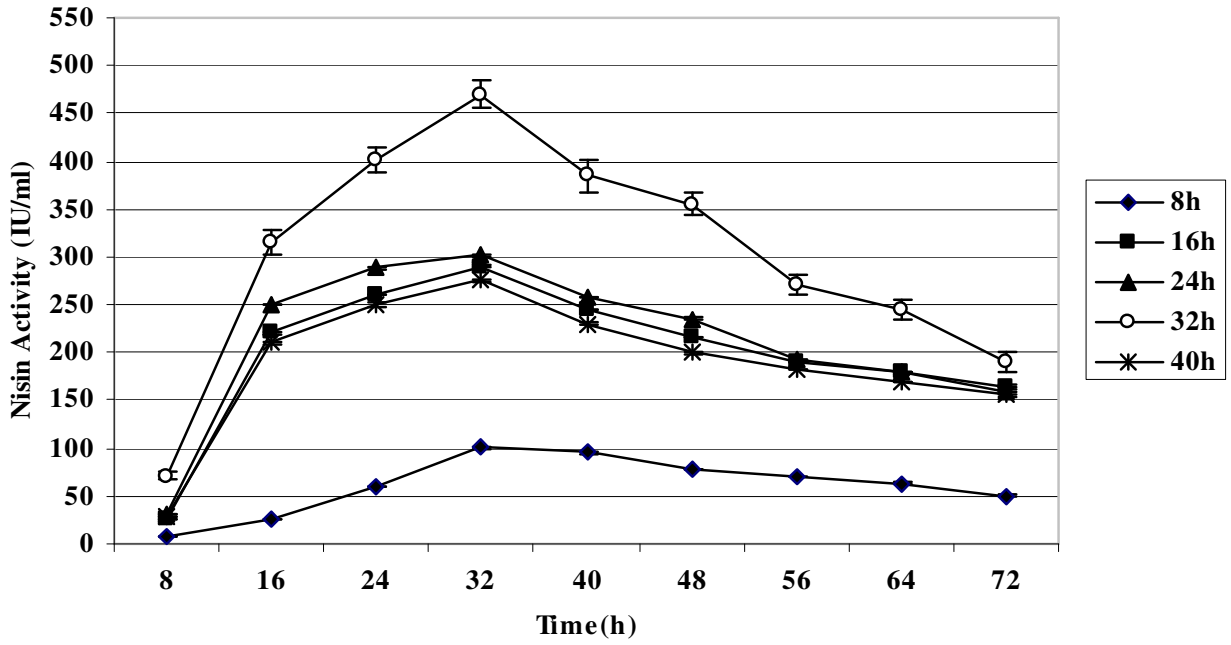

Figure 6. Effect of inoculum age on nisin production.

laboratory scale studies. Although 100 rpm (Penna and Moraes, 2002; Penna et al., 2005; Jozala et al., 2005) and 200 rpm (Guerra and Pastrana, 2001; Jozala et al., 2007) agitations were used in nisin production studies, Figure 3 shows that maximum nisin production and productivity were achieved at 200 rpm agitation (598 and 37.37 IU/ml/h) in contrast with the production at 100 rpm (450 and 18.75 IU/ml/h). It means a 32% increase in nisin production and a two-fold in productivity.

Different F/M ratios of 5.00 (Guerra and Pastrana,

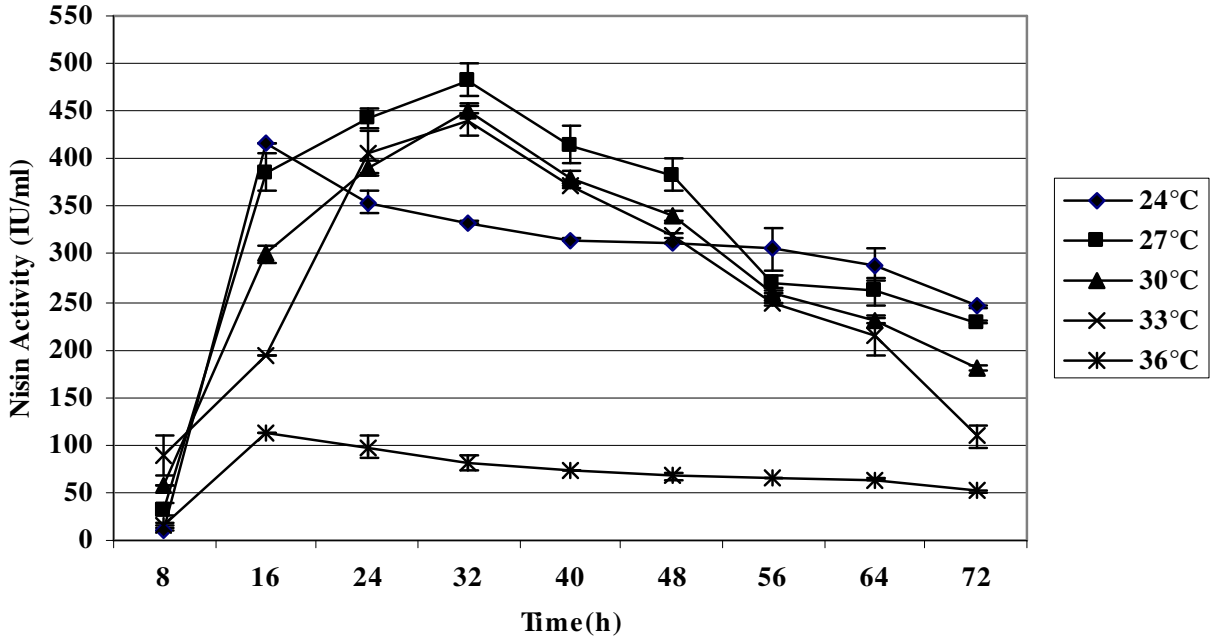

Figure 7. Effect of temperature on nisin production.

2001; Penna and Moraes, 2002; Penna et al., 2005), 2.50 (Liu et al., 2005b), 2.00 (Lv et al., 2005) and 1.33 (Jozala et al., 2007) were used in nisin production studies. Increasing F/M ratio had a meaningful effect on the nisin production (Figure 4). Nisin production was similarly affected by 10.00 and 5.00 F/M ratios (649 and 625 IU/ml) but maximum productivity was observed with 5.00 F/M ratio (31.25 IU/ml/h as compared to 27.68 IU/ml/h with 10.00 F/M ratio). Although *L. lactis* is a micro-aerophilic bacterium (Remaut et al., 2006), but our study showed that selection a proper agitation and F/M ratio is very important for maximum production and productivity.

Various inoculum sizes, 1% v/v (Lv et al., 2005), 2% v/v (Guerra and Pastrana, 2001), 4% v/v (Kim et al., 1997), 5% v/v (Liu et al., 2005b), 10% v/v (Penna and Moraes, 2002; Penna et al., 2005; Jozala et al., 2007) were utilized in nisin production studies. Our study showed that the highest nisin production and productivity was occurred at 7.5% v/v but inoculum size more than 2.5% v/v had a similar effect on the nisin production which is very important point for industrial scale up of nisin production. The lowest nisin production was seen with a 1% v/v inoculum size.

Figure 1 obviously demonstrated that after 32 h, cell density as well as cell count reached its maximum level; bacterial growth was at the end of logarithm phase of growth curve and maximum nisin production and productivity was obtained at this point. A 56% increase in nisin production was seen with 32-h-old inoculum age compare to 16, 24 and 40-h cultures. Different inoculum ages, for example, 8-h-old culture (Liu et al., 2005b), 12-h-old culture (Guerra and Pastrana, 2001; Lv et al., 2005), OD_{600nm} = 0.7 (Penna and Moraes, 2002) and 36-h-old culture (Penna et al., 2005; Jozala et al., 2007) were used by investigators.

L. lactis is a mesophilic bacterium (optimal growth temperature around 30°C) (Miyoshi et al., 2003) which can even grow at of 10°C, but not at 45°C (Samaržija et al., 2001). According to our study (Figure 7), 27°C was the optimum temperature for nisin production, in contrast to all investigations which were reported 30°C as incubation temperature for nisin production (Kim et al., 1997; Guerra and Pastrana, 2001; Penna and Moraes, 2002; Penna et al., 2005; Liu et al., 2005b; Lv et al., 2005; Jozala et al., 2005, 2007). Even the optimum temperature for nisin productivity was at 24°C (25.93 IU/ml/h at 24°C compared to 18.41 IU/ml/h at 27°C).

Because the main object of present investigation was evaluation of the non-nutritional factors effect on nisin production and their standardization, nisin purification, in order to measure retention time, was made. RP-HPLC analysis indicated that the purified peptide produced by *L. lactis* subsp. *lactis* ATCC 11454 and the standard nisin had identical retention times and chromatographic profile (Figure 8).

Conclusion

MRS and M17 media have been reported as suitable media for nisin production by *L. lactis* (Jozala et al., 2005). Evaluation of non-nutritional factors effect is necessary

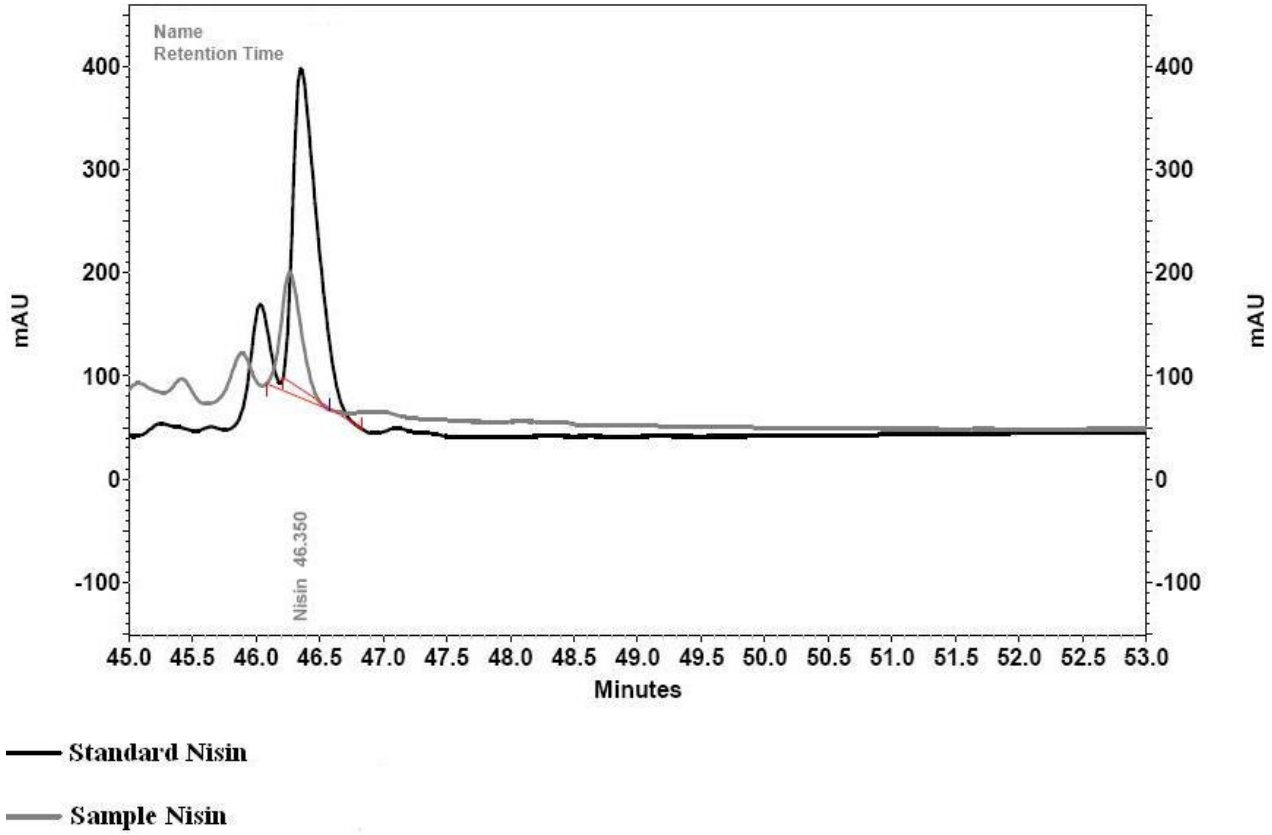

Figure 8. RP-HPLC analysis of standard nisin and nisin purified from *L. lactis* subsp. *lactis* ATCC 11454. Samples were eluted with the mobile phase consisting of 0.1% (v/v) trifluoroacetic acid (TFA) in a mixture of water (eluent A) and acetonitrile (eluent B). (Both reagents were HPLC grade and obtained from Merck, Germany). A gradient program was followed: samples were initially eluted with 100% A for 5 min, then with a linear gradient 0 – 50% B over 45 min, followed by a linear gradient to 100% B over 5 min and maintained at 100% B for 7 min. The flow rate was maintained at 1 ml/min, absorbance was monitored at 215 nm using K-2501 UV detector (Knaure, Germany) and the column was kept at a constant temperature of 35°C. Data analysis was performed using a chromatography software package (ChromGate, version 3.1, Knaure, Germany). Black curve: standard nisin (Sigma); Gray curve: nisin purified from the culture medium.

for optimization of nisin production. This study showed that even in standard medium (MRS), nisin production is strongly influenced by non-nutritional factors. Optimization of these factors can increase nisin production and productivity without any changes in components concentration or adding any supplements into the medium. In order to optimize MRS medium for maximum nisin production and productivity and after evaluation of the effect of non-nutritional factors on nisin production, utilization of a statistically experimental design technique is suggested.

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REFERENCES

Anton R, Barlow S, Boskou D, Castle L, Crebelli R, Dekant W, Engel K, Forsythe S, Grunow W, Heinonen M, Chr. Larsen J, Leclercq C, Mennes W, Rosaria Milana M, Pratt I, Rietjens I, Svensson K, Tobback P, Toldrá F (2006). Opinion of the scientific panel on food Additives, flavourings, processing aids and materials in contact with food on a request from the commission related to the use of nisin (E 234) as a food additive. Eur. Food Safety Authority J. 314: 1-16. Available from:
http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/afc_op_ej314_nisin_op_en1,0.pdf

Arauz LJ, Jozala AF, Mazzola PG, Vessoni Penna TC (2009). Nisin biotechnological production and application: a review. Trends Food Sci. Technol. 20: 146-154.

Bunthof CJ, Van Den Braak S, Breeuwer P, Rombouts F, Abee T (1999). Rapid fluorescence assessment of the viability of stressed *Lactococcus Lactis*. Appl. Environ. Microbiol. 65: 3681-3689.

Chollet E, Sebti I, Martial-Gros A, Degraeve P (2008). Nisin preliminary

study as a potential preservative for sliced ripened cheese: NaCl, fat and enzymes influence on nisin concentration and its antimicrobial activity. Food Control 19: 982-989.
Cleveland J, Montville TJ, Nes IF, Chikindas ML (2001). Bacteriocins:safe, natural antimicrobials for food preservation. Int. J. Food Microbiol. 71: 1-20.
Daoudi L, Turcotte C, Lacroix C, Fliss I (2001). Production and characterization of anti-nisin Z monoclonal antibodies: suitability for distinguishing active from inactive forms through a competitive enzyme immunoassay. Appl. Microbiol. Biotechnol. 56: 114-119.
Deegan LH, Cotter PD, Hill C, Ross P (2006). Bacteriocins: Biological tools for bio-preservation and shelf-life extension. Int. Dairy J. 16: 1058-1071.
Devlieghere F, Vermeiren L, Debever J (2004). New preservation technologies: Possibilities and limitations. Int. Dairy J. 14: 273-285.
Guerra NP, Pastrana L (2001). Enhanced nisin and pediocin production on whey supplemented with different nitrogen sources. Biotechnol. Lett. 23: 609-612.
Jozala AF, Novaes LCL, Cholewa O, Moraes D, Vessoni Penna, TC (2005). Increase of nisin production by *Lactococcus lactis* in different media. Afr. J. Biotechnol. 4: 262-265.
Jozala AF, Andrade MS, Arauz LJ, Pessoa Jr. A, Vessoni Penna TC (2007). Nisin production utilizing skimmed milk aiming to reduce process cost. Appl. Biochem. Biotechnol. 136: 515-528.
Kim WS, Hall RJ, Dunn NW (1997). The effect of nisin concentration and nutrient depletion on nisin production of *Lactococcus lactis*. Appl. Microbiol. Biotechnol. 48: 449-453.
Laridi R, Kheadr EE, Benech RO, Vuillemard JC, Lacroix C, Fliss I (2003). Liposome encapsulated nisin Z: optimization, stability and release during milk fermentation. Int. Dairy J. 13: 325-336.
Liu X, Chung Y, Yang S, Yousef AE (2005a). Continuous nisin production in laboratory media and whey permeate by immobilized *Lactococcus lactis*. Process Biochem. 40: 13-24.
Liu C, Liu Y, Chen S (2005b). Effects of nutrient supplements on simultaneous fermentation of nisin and lactic acid from cull potatoes. Appl. Biochem. Biotechnol. 121: 475-83.
Lv W, Zhang X, Cong W (2005). Modelling the production of nisin by *Lactococcus lactis* in fed-batch culture. Appl. Microbiol. Biotechnol. 68: 322-326.
Miyoshi A, Rochat T, Gratadoux JJ, Loir Y, Oliveira SC, Langella P, Azevedo V (2003). Oxidative stress in *Lactococcus lactis*. Genet. Mol. Res. 2: 348-359. Available from: http://www.funpecrp.com.br/GMR/ year2003/vol4-2/pdf/gmr0075.pdf.
Penna VTC, Moraes DA (2002). Optimization of nisin production by *Lactococcus lactis*. Appl. Biochem. Biotechnol. 98: 775-89.
Penna VTC, Jozala AF, De Lencastre NLC, Pessoa Jr A, Cholewa O (2005). Production of nisin by *Lactococcus lactis* in media with skimmed milk. Appl. Biochem. Biotechnol. 121: 619-37.
Pirad JC, Muriana PM, Desmazeaud MJ, Klaenhammer TR (1992). Purification and partial characterization of lacticin 481, a lanthionine-containing bacteriocin produced by *lactococcus lactis* subsp. *lactis* CNRZ 481. Appl. Environ. Microbiol. 58: 279-284.
Pongtharangkul T, Demirci A (2004). Evaluation of agar diffusion bioassay for nisin quantification. Appl. Microbiol. Biotechnol. 65: 268-272.
Pongtharangkul T (2006). Enhanced nisin production in a biofilm reactor and separation of nisin. PhD dissertation, The Pennsylvania State University, Pennsylvania, USA.
Remaut E, Braat H, Vandenbroucke K, Rottiers P, Steidler L (2006). Clinical potential of *Lactococcus lactis* mediated delivery of human interleukin-10 and trefoil factors. Biosci. Microflora. 25: 81-97.
Roukas T (2006). Biotechnology of citric acid production. In: Shetty et al. (eds.) Food Biotechnology, Second edition, CRC Press, USA, p. 367.
Samaržija D, Antunac N, Havranek JL (2001). Taxonomy, physiology and growth of *Lactococcus lactis*: a review. Mljekarstvo. 51: 35-48.
Strobel RJ, Sulivan GR (1999). Experimental design for improvement of fermentations. In: Demain and Davis (eds.) Manual Of Industrial Microbiology and Biotechnology, Am. Soc. Microbiol. Press, USA, pp. 80-82.
Vázquez JA, Cabo ML, González MP, Murado MA (2004). The role of amino acids in nisin and pediocin production by two lactic acid bacteria A factorial study. Enzyme Microb. Technol. 34: 319-325.