African Journal of Biotechnology Vol. 10(66), pp. 14920-14925, 24 October, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.979
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Processing of byproducts to improve nisin production by *Lactococcus lactis*

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Accepted 29 June, 2011

In the last years, disposal from dairy industries have received a special attention due its polluting power in the environment. For this reason, studies have obtained a positive support to develop different alternatives to recycle milk whey components. One of them is its utilization as culture media, aiming to produce biomolecules with noble applications. Nisin is an extracellular peptide, produced by *Lactococcus lactis*, this peptide has been applied as a natural additive once it presents broad antibacterial activity. Applications of this bacteriocin include dental care products, pharmaceutical products such as stomach ulcers and colon infection treatment and potential birth control. In batch cultures, *L. lactis* was performed in two different groups of assays. The first group milk whey was prepared in distilled water in four different concentrations: 100 g/l (S100); 50 g/l (S50); 30 g/l (S30); 10 g/l (S10). In the second group of assays, two supplements were added in milk whey with concentration 100 g/l (S100): (1) 5 g/l yeast extract (A1); (2) 5 g/l yeast extract and 10 ml (v/v) tomato extract. Nisin activity was assayed through agar diffusion utilizing *Lactobacillus sakei*. The results show that the utilization of powder milk whey with concentration of 100 g/l can be used as a culture medium with supplementation. This media is favorable to develop *L. lactis* cells and nisin production, reaching an activity of about 4 logAU. Biological processing of milk byproduct can be considered as one of the profitable utilization alternatives, generating high-value bioproducts and stimulates researches for its use.

Key words: Nisin, byproducts, *Lactococcus lactis*, batch culture, powder milk whey.

INTRODUCTION

Nisin, antimicrobial peptide (3.4 kDa), is produced by *Lactococcus lactis* ATCC 11454 during its exponential growth phase (Vessoni Penna and Moraes, 2002). Nisin is a bacteriocin commercially used as natural agent for food biopreservation. It has recently been considered safe by the World Health Organization (WHO) and by the Food and Drug Administration (FDA), with the denomination of generally recognized as safe (GRAS) (de Vuyst and Vandamme, 1994; Arauz et al., 2009).

This bacteriocin has large antimicrobial activity spectrum against Gram-positive bacteria and their spores, but shows little or no activity against Gram-negative bacteria, yeasts or moulds. However, Gram-negative bacteria can be sensitized to nisin by exposing to chelating agents (EDTA), sublethal heat and freezing (Vessoni Penna et al., 2006).

As a result of its antimicrobial properties, nisin has been accepted as a safe and natural preservative in different areas of food industry and it has also been used as treatment for some health conditions such as stomach and colon ulcers, cosmetic and veterinary products (Delves-Broughton et al., 1996; Liu et al., 2004; Von Sataszewski and Jagus, 2008).

The production of bacteriocins is normally performed in complex growth media: Man Rugosa and Sharpe (MRS), all purpose with Tween (APT), Elliker, brain heart infusion (BHI), tryptone glucose extract (TGE), trypticase soy

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broth (TSB) and trypticase soy broth yeast extract (TSBYE). Although these media promote exuberant growths and have relatively high bacteriocin levels, they have high cost that makes them unsuitable for a large-scale production (Guerra et al., 2001). Furthermore, some medium components (for example, large amounts of proteins, which are not totally consumed by the producer strains at fermentation end) may interfere with the subsequent bacteriocin purification (Barefoot and Klaenhammer, 1984).

Although the titers of bacteriocin were lower than those obtained on MRS broth, Guerra et al. (2001) demonstrated the feasibility of the production of nisin and pediocin in an effluent of low cost. The kinetic characterization of both nisin and pediocin production helps to clarify the different effects produced by the nutrient sources (lactose, glycine and potassium phosphate bibasic) used in their work. In addition, the models developed allowed a good construction of the experimental data and identified the key variables in the production of both nisin and pediocin.

Nowadays, whey is a major byproduct of modern cheese and casein industries. On average across the world, volumes of whey are growing at about the same rate as milk volumes (42% per year; FAO, 2006). This increased quantity of milk is being channeled into the production of larger volumes of cheese, casein/caseinate and other dairy products, resulting in concomitant increases in the whey volume (Smithers, 2008).

Milk whey is a byproduct of dairy industry and contains rich nutrients as lactose, soluble proteins and minerals salts. Unfortunately, whey and its associated nutritional qualities have traditionally been treated as waste and represent an important disposal and pollution issue because of its high biological and biochemical oxygen demand (Liu et al., 2004). Consequently, it is interesting to use this byproduct as fermentation medium for the production of value-added products (Arauz et al., 2009).

The utilization of the milk whey as culture media has been employed in different studies. Guerra et al. (2001) utilized milk whey to produce two different bacteriocins: nisin and pediocin, from *Lactococcus lactis* and *Pediococcus acidilactici,* respectively, in batch cultures for 18 h. They observed higher nisin production in diluted whey (22.9 BU/ml) (mixed with wash waters) (BU: bacteriocin units is the amount of bacteriocin needed to obtain 50% of growth inhibition measured spectrophotometrically at 700 nm) in relation to concentrated whey (liquid remaining after the first cheese pressing) which was 8.3 BU/ml. In another study, Guerra et al. (2007) utilized diluted milk whey with supplements to produced pediocin from *P. acidilactici*.

Flores and Alegre (2001) using supplemented whey during batch fermentation, obtained a maximum nisin activity of 5280 AU/ml after 9 h of process (pH 4.9). Mondragon-Parada et al. (2006) verified that supplemented filtered whey enhanced the biomass production of lactic acid bacteria. Some researchers applied a mixed culture of *L. lactis* and *Saccharomyces cerevisiae* to whey-based medium to stimulate the production of nisin (Liu et al., 2006).

Arauz et al. (2008) utilized three different media with milk whey; (1) without filtration and pH 6.8, adjusted with NaOH 1 M; (2) without pH adjustment, and autoclaved at 121°C for 30 min; (3) filtered (1.20 and 0.22 µm membrane filter). Nisin activity was higher in milk whey without filtration with pH adjusted to 6.8, and was around 444805.35 AU/ml.

In this research we studied the nisin production in several media through different concentration of powdered milk whey, with or without supplementation.

MATERIALS AND METHODS

Bacterial strains

L. lactis ATCC 11454, nisin producer strain and *Lactobacillus sakei* ATCC 15521, bioindicator of nisin activity, were used in this study. Both microorganisms were maintained at - 60°C in MRS broth (Man Rugosa Shepeer-Bacto Lactobacilli MRS broth, DIFCO) with 40% (v/v) of glycerol (Vessoni Penna et al., 2005; Jozala et al., 2007).

Growth media preparation

In previous work, the liquid milk whey mixed with wash water was exploited to nisin production (Arauz et al., 2008). In this study , the growth media utilized in the assays for nisin production, was powder milk whey (generously provided by a local dairy plant, Lactogal, Produtos Alimentares S.A., Portugal). The first group of assays milk whey was prepared in distilled water in four different concentrations: 100 g/l (S100); 50 g/l (S50); 30 g/l (S30) and 10 g/l (S10). In the second group of assays, two supplements were added to the milk whey with concentration of 100 g/l (S100): (1) 5 g/l yeast extract (A1); (2) 5 g/l yeast extract and 10 ml (v/v) tomato extract (A2). The growth media pH was adjusted to 6.8 with NaOH 0.1 M and sterilized at 121°C for 30 min.

Batch cultures

Pre-culture was prepared through the cultivation of the 100 µl of stock culture *L. lactis* (10^7 CFU/ml) inoculated into 50 ml of MRS broth in 250 ml flask on rotatory shaker under the follow conditions: 100 rpm, at 36 h and 30°C. After the 36 h, 5 ml aliquot of pre-culture growth were transferred into 50 ml of milk whey medium (S100, S50, S30, S10, A1 and A2) in 250 ml flask which were incubated for another period of 36 h in the same conditions described earlier. Each transfer was repeated for 5 times.

After each incubation period, 5 ml was collected aseptically for pH, cell growth and nisin activity analysis. Gram technique was applied to monitor contamination (Arauz at al., 2008; Jozala et al., 2007).

Nisin activity

Nisin activity was evaluated by agar diffusion method utilizing *L. sakei* as sensitive microorganism. *L. sakei* was grown in MRS broth and incubated in rotator shaker (100 rpm, 30°C and 24 h). For bioassay, the medium was composed of 0.8 g of agar (Bacto agar,

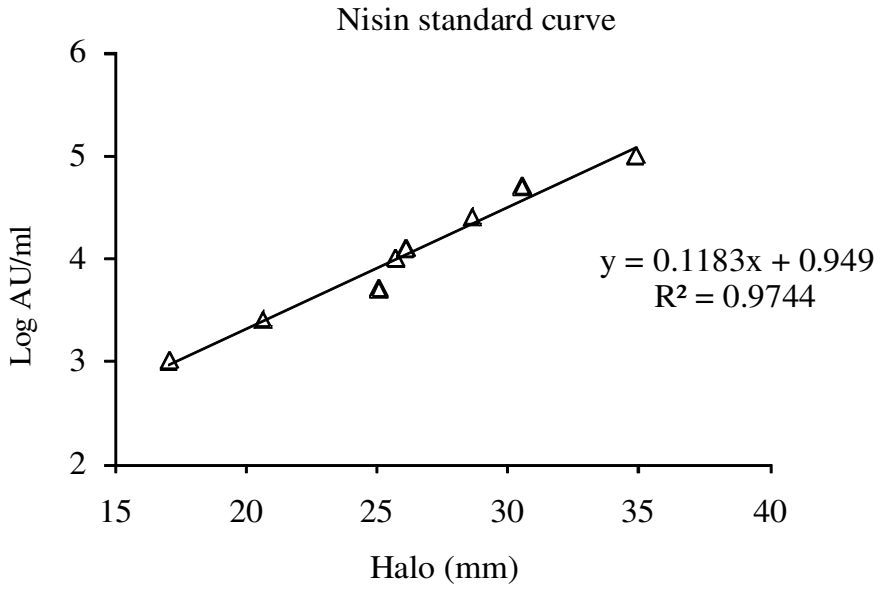

Figure 1. Standard curve of nisin. The relation between arbitrary units (AU/ml) and halo diameter (H, mm) was determined using concentrations of standard nisin (10^1 to 10^5 AU/ml). Based on calibration curves between AU/ml and IU/ml, 1.09 ± 0.17AU corresponded to 1.0 IU (40 IU = 1 µg of pure nisin).

Difco) for each 100 ml of MRS broth. After autoclaving the agar medium, it was cooled around 40°C and inoculated with *L. sakei* (10^6 CFU/ml). Each 20 ml was aseptically poured in sterile Petri dishes (100 × 15 mm) and after solidified, 3 mm wells were cut out with a sterile pipe of 5 mm diameter. Samples from batch culture were centrifuged at 13201× *g* for 10 min at 10°C and the supernatant collected was filtered through a 0.22 µm membrane filter; 50 µl of supernatant was transferred into the wells of the *L. sakei* inoculated agar. The plates, without inversion were incubated at 30°C for 24 h. Afterwards, the diameters of the growth inhibition zones were measured in four directions and the average diameters (±5 mm) of the halos were related to arbitrary units (AU/ml). The results were compared with a nisin standard curve.

For the standard curve (Figure 1), a nisin stock solution was prepared by adding 1 g of commercial nisin (Nisin®, Sigma, St. Louis, MO - standard at an activity of 10^6 AU, containing 25000 µg of nisin per gram) into 10 ml of 0.02 mol/l HCl. The relation between arbitrary units (AU/ml) and halo diameter (H, mm) was determined using concentrations of standard nisin (10^1 to 10^5 AU/ml). Based on calibration curves between AU/ml and IU.ml^{-1}, 1.09 ± 0.17AU corresponded to 1.0 IU (40 IU = 1 µg of pure nisin). The activity of nisin expressed in AU/ml was converted to mg.l^{-1}, through the relation: Nisin (mg/l) = (z × 0.025), where *z* = AU/ml and 0.025 is a conversion factor related to 2.5% of real nisin (Barefoot and Klaenhammer, 1984; Vessoni Penna et al., 2006; Jozala et al., 2007; Pongtharangkul and Demirci, 2004).

RESULTS AND DISCUSSION

The influence of the milk whey in the behavior of *L. lactis* was analyzed taking 5 ml of the culture of each transfer process, as previously reported in our earlier works (Vessoni Penna et al., 2005, 2006; Jozala et al., 2005, 2007).

The pre culture of *L. lactis* in MRS broth showed the activities of 3.27 and 3.37 logAU, that is equals to 46.53 and 58.17 mg/l, respectively, with a growth of approximately 10^6 CFU/ml; both used to initiate the culture with milk whey. The growth and nisin formation, in the pre culture, is due to the fact that, MRS broth contained complex nutrients, keeping ideal conditions for the start of the assays in the culture media of milk whey and supplements (Table 1), named as S100, S50, S30, S10, A1 and A2.

The medium S100 showed *L. lactis* growth in all transfers, and nisin activity was detected only in the 2nd and 5th transfer, corresponding to 2.56 and 3.33 logAU, which was equal to 9.12 and 53.93 mg/l, respectively. The same behavior was noted in the medium S50; the nisin activity was detected only in the 1st and 5th transfers, corresponding to 2.59 and 2.65 logAU which was equal to 9.83 and 11.23 mg/l, respectively. In both assays, S100 and S50, pH values were around 5.4.

For assays S30 and S10, culture medium were more diluted than that of S100 and S50 and some nutrients are necessary to microorganism development gotten in smaller concentration. For this reason, the culture medium proportioned to the *L. lactis* cells was not a favorable environment for the growth.

Although the media S100 was the best conditions for the development of *L. lactis* cells and the nisin release (Table 2), compared with the results giving by the media S10 and S30, it was impossible to detect linearity in the activities. This could be associated with the absence of nutrients in the culture media, as reported in the work of Jozala et al. (2007) who observed that the concentration equilibrium of the nutrients in a medium was essential for

Table 1. Culture media in different concentrations of milk whey and supplements (g/l).

Culture	S10	S30	S50	S100	A1	A2
Milk whey	10	30	50	100	100	100
Yeast extract	-	-	-	-	5	-
Tomato extract	-	-	-	-	-	10

- Not added.

Table 2. Nisin activity and concentration in each transfer of the *L. lactis* cells in batch culture utilizing different concentrations of milk whey under the conditions of 100 rpm at 30°C for 36 h.

	Transfer	CFU/ml	pH	Halos (mm)	[a]AU/ml	Log (AU)	[b]mg/l
Milk whey (100 g/l) (S100) initial pH: 6.6	Pre culture (MRS)	2.5×10^6	4.89	19.62	1861.02	3.27	46.53
	1	1.3×10^4	5.41	-	-	-	-
	2	8.7×10^4	5	13.64	364.77	2.56	9.12
	3	1.9×10^5	5.58	-	-	-	-
	4	2.13×10^5	5.68	-	-	-	-
	5	4.2×10^5	5.44	20.16	2157.39	3.33	53.93
Milk whey (50 g/l) (S50) initial pH: 6.6	Pre culture (MRS)	2.5×10^6	4.89	19.62	1861.02	3.27	46.53
	1	1.7×10^4	5.45	13.91	393.15	2.59	9.83
	2	3.2×10^4	5.33	-	-	-	-
	3	1.7×10^5	5.76	-	-	-	-
	4	1.8×10^5	5.59	-	-	-	
	5	2.2×10^5	5.68	14.40	449.28	2.65	11.23
Milk whey (30 g/l) (S30) initial pH: 6.6	Pre culture (MRS)	2.5×10^6	4.82	19.62	1861.02	3.27	46.53
	1	-	4.33	-	-	-	-
	2	-	5.2	-	-	-	-
	3	-	5.26	-	-	-	-
	4	-	5.39	-	-	-	-
	5	-	5.5	-	-	-	-
Milk whey (10 g/l) (S10) initial pH: 6.6	Pre culture (MRS)	2.5×10^6	4.82	19.62	1861.02	3.27	46.53
	1	-	4.1	-	-	-	-
	2	-	5.33	-	-	-	-
	3	-	5.76	-	-	-	-
	4	-	5.59	-	-	-	-
	5	-	5.68	-	-	-	-

[a]Arbitrary units per milliliter: AU/ml $=10^{(0.1183 \times H + 0949)}$; H = diameter of the halo (mm); [b]Nisin concentration: mg/ml = (x) *0.025, in which x= activity (AU/ml) and is a conversion value related with 2.5% of pure nisin.

the higher nisin productivity by *L. lactis* cells.

Besides, the lactic bacteria are fastidious and require culture media with high nutritional value, which increase the growth and the bacteriocin production. Many studies describe the MRS and M17 broth as great culture media used for cellular growth and bacteriocin production by the *L. lactis* (Biswas et al., 1991; Toba et al., 1991; Brink et al., 1994; Reid et al., 1998; Cheigh et al., 2002)

Guerra and Pastrana (2002) observed that nutrient sources were not adequate to increase bacteriocin production on diluted whey. Further studies based on the search for other sources (like yeast extract or peptones)

Table 3. Nisin activity and concentration in each transfer of the *L. lactis* cells in batch culture utilizing different concentrations of milk whey supplemented under the conditions of 100 rpm at 30°C for 36 h.

Transfer	CFU/ml	pH	Halos (mm)	[a]AU/ml	Log (AU)	[b]mg/l
Milk whey (100 g/l) with yeast extract 5 (g/l) (A1) initial pH: 6.3						
Pre culture (MRS)	2.5×10^6	4.96	20.44	2326.78	3.37	58.17
1	1.7×10^4	4.55	16.09	711.46	2.85	17.79
2	3.2×10^4	5.15	-	-	-	-
3	1.7×10^5	5.04	16.57	810.84	2.91	20.27
4	1.8×10^5	4.96	16.59	815.27	2.91	20.38
5	2.2×10^5	4.65	17.91	1167.24	3.07	29.18
Milk whey (100 g/l) with yeast extract (5 g/l) and 10 ml tomato extract (v/v) (A2) initial pH: 6.1						
Pre culture (MRS)	2.5×10^6	4.96	20.44	2326.78	3.37	58.17
1	1.2×10^3	4.53	15.04	534.49	2.73	13.36
2	1.5×10^4	5.16	17.73	1113.67	3.05	27.84
3	1.6×10^4	4.98	16.55	806.43	2.91	20.16
4	1.8×10^4	4.89	-	-	-	-
5	2.0×10^4	4.54	17.31	993.27	3.00	24.83

[a]Arbitrary units per milliliter: AU/ml $=10^{(0.1183 \times H + 0949)}$, in which H = diameter of the halo (mm). [b]Nisin concentration: mg/ml = (x) * 0.025, in which x= activity (AU/ml) and is a conversion value related with 2.5% of pure nisin.

are needed to optimize the unbuffered whey composition.

In additional, the same authors utilized yeast extract, lactose and glucose for supplements into the milk whey for bacteriocin production. They observed that the use of feeding substrates containing glucose instead of lactose could be an appropriate alternative for increasing fed-batch production of pediocin (Guerra et al., 2007).

Jozala et al. (2007) showed that nutritional components of skimmed milk diluted with 2.27 g total solids (maximum of nisin 501.93 mg/l), influenced the nisin expression by the cells during the cultivation, suggesting that milk whey could increase nisin production and reduced the cost of production of this biomolecule.

According to the research, the maximum nisin values obtained in that study in milk whey not filtered (11120.13 mg/l) was 22 fold higher than the milk whey diluted. Since the culture media is of low cost, the higher nutritional content of milk and milk whey gave an excellent growth condition for the *L. lactis* and nisin expression.

Observing the results obtained in milk whey, we choose to use nutritional supplements. By the analysis of the bibliography, we tried to put in this study culture media that promote the growth and nisin excretion by the microorganism. Based on the ATCC site: (http://www.lgcstandardsatcc.org/LGCAdvancedCatalogueSearch/ProductDescription/tabid/1068/Default.aspx) the ideal culture media for the development of *L. lactis* cells contain yeast extract, tomato juice and milk. Because of this information, two culture media containing, separately, yeast extract and tomato extract utilization was elaborated as base culture media; S100 in this study (Table 1).

The supplementations of the media gave a better adaptation for the nisin producing cells. We observed in the transfers, a higher linearity between the results. In the A1 medium containing yeast extract, the nisin activity observed in the 1st transfer was 2.85 logAU, corresponding to 17.79 mg/l, pH 4.55 and cellular growth of approximately 10^4 log CFU/ml. Higher activity was observed in the 5th transfer as 3.07 logAU corresponding to 29.18 mg/l, pH 4.65 and cellular growth of approximately 10^5 log CFU/ml. In the 2nd transfer, nisin activity was not detected and could be related to the pH value of 5.15 (Table 3).

In the A2 medium, containing the tomato extract, the nisin activity was observed during the first three transfers, giving the values 2.73, 3.05 and 2.91 logAU, respectively for the first, second and third transfers. In the 5th transfer, the nisin activity was 3 logAU, at pH 4.54 with cellular growth of 10^4 CFU/ml.

The cellular growths were stable during all the process. However, nisin activity was not detected in the 4th transfer which can be attributed to the increase of the pH. This phenomena occurred in the assay with A1 medium; the 2nd transfer did not show nisin activity and the pH value was similar to the pH of the 4th transfer of the A2 medium assay, around 5.15 (Table 3). Comparing the assay A1 and A2, both media showed the same results.

Nisin production is affected by several cultural factors such as producer strain, nutrient composition of media, pH, temperature, agitation and aeration, and also by other factors, such as substrate and product inhibition, adsorption of nisin onto the producer cells and enzymatic degradation (Chandrapati and O´Sullivan, 1998).

In this study for all assays, no decrease in nisin titers was observed and the highest nisin activity was detected in pH < 5. In pH values smaller than 6, at least, 80% of the nisin expressed by the cells were released in the culture medium. On the other hand, in pH higher than 6, most of the nisin could be retained in the cellular membrane or be inside the cells (Chandrapati and O´Sullivan, 1998; Parente and Ricciardi, 1999; Yang et al., 1992).

Conclusion

Although the work was developed by Arauz et al. (2008), the utilization of milk whey without the supplementation of nutrients was showed to be a great medium for the nisin formation and dispersion by *L. lactis*. The results showed that the utilization of powder milk whey can be used as a fermentation medium with supplementation. The supplementation was essential for the formation and dispersion of nisin by *L. lactis*; in this case, the higher nisin concentration is related to nitrogen fonts present in the medium.

The hypotheses for these results could be associated with the kind of treatment that was probably used to obtain the milk whey. Biological processing of byproducts (milk whey) can be considered one profitable alternative, generating high-valued bioproducts. Using this low-cost media on the microbial cultures promotes economical advantages, reducing environment pollution and stimulates researches for the use of this byproduct. Further studies based on different powder milk whey concentration with supplementation should be delineated.

ACKNOWLEDGEMENT

The authors wish to thank the Brazilian Committees for the Scientific Technology Research (CNPq, FAPESP, CAPES), for financial support and scholarship.

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