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Characterization of Nisin Produced by *Lactococcus lactis*

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ABSTRACT

Lanthionine-containing antimicrobial peptides are a growing family of compounds which have received the name lantibiotics, these peptides are unique in that they are ribosomally synthesized as prepeptides and undergo post translational processing of number of amino acids (serine, threonine and cysteine) into dehydro residues and thioether crossbridges. Nisin gene was chromosomal encoded. Nisin produced by *Lactococcus lactis* Fc2 was purified and characterized. Purified nisin at pH 5 and 6 level stable and nisin was relatively heat stable from 40 to 90°C for 30 min. SDS-PAGE was used to detect nisin, which corresponded to an apparent molecular mass of about 3.5 kDa. It identified as a 7 kDa peptide after 2 weeks storage. Analysis of amino acids of purified nisin exhibited cystiene in composition.

Key Words: Nisin; *Lactococcus lactis*; SDS-PAGE; Amino acids; Plasmids

INTRODUCTION

Lactic acid bacteria (LAB) are industrially important organisms recognized for their fermentative ability as well as their health and nutritional benefits. The ability of LAB to produce antimicrobial substances has long been used to preserve foods (Yang, 2000)**.** Bacteriocins are antimicrobial proteinaceous compounds that are generally inhibitory towards sensitive strains and are produced by both Gram-positive and Gram-negative bacteria (Yildirim & Johnson, 1997). Nisin is the most extensively characterized bacteriocin of antimicrobial proteins produced by lactic acid bacteria **(**Jack *et al*., 1994**).**

Nisin is heat stable; however, bacteriocins differ considerably in their heat stability and many of them can withstand temperatures range between 60-100°C for more than 30 minutes **(**Jack *et al*., 1994**).** The essential proteins of some bacteriocins contain unusual amino acids *e.g*. α, β-unsaturated amino acids (lantibiotics), thioether amino acids (lantibiotics) or several cross-linkages *e.g*. disulfide bridges (Klaenhammer *et al.,* 1994). Nisin is the only bacteriocin that is accepted as a food preservativeand has a broad spectrum of activity against Gram-positive organisms including spore forming bacteria. Nisin occurs as subtypes, A, B, C, D or E, that differ in amino acid composition and biological activity (Hurst, 1981). It is a pentacyclic cationic polypeptide, refered to as a lantibiotic. Nisin contains 34 amino acids and is synthesized by post-translational processing of ribosomally synthesized precursors (Hansen & Liu, 1990**).** The main purpose of this study was to characterize the nisin produced by *Lactococcus lactis* Fc2.

MATERIALS AND METHODS

Microorganisms. *Lactococcus lactis* Fc2 was isolated from farmed cheese in our laboratory and gave the highest nisin production and *Lactococcus lactis* 11454 (Abdel Karem *et al*., 2005**).**

Factors affecting the stability of nisin. pH of the cell free supernatant (CFS) was adjusted at 3, 4, 5, 6, 7 & 8 and then nisin activity determined against *Micrococcus luteus, Bacillus cereus* and *Staphylococcus aureus.* Likewise temperature of CFS was adjusted at 10, 20, 30, 40, 50, 60, 70, 80, 90 & 100°C then determine nisin activity against *Micrococcus luteus*.

Detection of nisin. Broth media of Fc2 strain was sampled by adjusted the pH to approximately 2-3 with concentrated HCl, placed into boiling water bath for 5 min to remove cell bound nisin by hot acid extraction. Broth media were centrifuged at 12000x*g* for 12 min at 4°C to exclude the inhibiting activity due to acids. The pH of the CFS was adjusted to 6.5 with 1M NaOH and filter sterilized through cellulose membrane (0.45μm) (Tramer & Fowler, 1964).

Quantitative estimation of nisin prepared from the CFS. This was done after autoclaving and cooling the assay medium to about 45°C (Wolf & Gibbons, 1995). Nisin activity was carried out by critical dilution assay (Pucci *et al*., 1988). Serial two fold dilutions were made from CFS of the *Lactococcus lactis* culture from each dilution 40 μL was taken on the surface of filter paper disks on LTB agar plates and adjusted its pH at 6.5 with phosphate buffer 1% (v/v). Also LTB was seeded with 1% (v/v) suspension of log phase cells of every indicator strains culture. *Micrococcus luteus,* as indicator, was inoculated in plates and incubated at the optimum growth temperature.

Polyacrylamide gel electrophoresis. The crude nisin extracted in MRS media from *Lactococcus lactis* ATCC/11454, *Lactococcus lactis* Fc2 (at different doses of radiation 0.0, 1, 1.5, 2, 2.5, 3, & 4) and nisin standard powder. All these samples were subjected to sodium

dodyecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 20% uniform-pore gel. The gel was stained for protein with coomassie brilliant blue, along with the marker proteins (Lewus & Montville, 1991).

Amino acid composition for nisin produced by MRS media and whey media. To determine the amino acid composition of nisin standard, nisin produced in MRS and in whey media, 0.15 g from each were used. A suitable volume of sodium citrate buffer (pH 2.2) was added to the dried film of the hydrolyzed sample. After making all solutions, the samples were ready for analysis (Winder & Eggum, 1966). Amino acids were analyzed from each sample using high performance amino acid analyzer (Biochrom 20 Pharmacia Biotech).

Isolation of plasmids. Plasmids were isolated from *Lactococcus lactis* Fc2, *Lactococcus lactis* Fc2 irradiated at 1.5 kGy and at 2 kGy and *Lactococcus lactis* ATCC/ 11454 in MRS media as follows. Bacterial cells were grown overnight (12h) in 10 mL of MRS, centrifuged at 6000 g for 10 min at 4°C, wash the cells with 1 mL of SET buffer (resuspend and recentrifuge for 1 min), resuspend cells in 100 µL lysozyme 28 mg mL^{-1} and shaking well and then added 4 µL lytic and mixed well. The lytic mix was reacted for 2h at 37°C until solution become viscous. After that 180 µL 3 M precooled potassium acetate was added and place on ice for at least 20 min. Then centrifuged for 10 min at 8000 rpm and took the supernatant. Finally, precipitated the supernatant by equal volume of isopropanol and centrifuged at 10000 rpm for 10 min, washed with 1 mL 70% ethanol and centrifuge again at 10000 rpm for 5 min (decant supernatant). Dried for 10 min at room temperature and resuspended in 10 µL TE buffer (Klaenhammer, 1984).

RESULTS AND DISCUSSION

Effect of pH on nisin stability. Nisin activity depended on initial pH as it affects the growth of the organism. The optimum pH for bacteriocin production has been shown to be affected by the culture media and species (Chinachoti *et al*., 1997: Zamfir *et al*., 2000). Nisin activity was at lower level in acidic and alkaline pH. At pH 6 and 7 nisin activity was at higher values and stability recorded 10000 Au/mL^{-1} against *Micrococcus luteus* (Table I). The greater inhibitory effect of nisin at acid than at neutral pH was probably due mainly to the greater solubility of nisin at acidic pH (Hurst & Hoover, 1983). On the other hand, activity of nisin was higher at pH 5 and 6 than other pH values tested against *Staphylococcus aureus* and *Bacillus cereus*. Similar results were obtained by (Gross & Morell, 1971; Hurst, 1981; Matsusaki *et al.,* 1996 & Amiali *et al*., 1998).

Effect of temperature on nisin stability. The production of bacteriocin by lactic acid bacteria is known to be temperature dependent. The optimum incubation temperature for bacteriocin varies among different strains of lactic acid bacteria **(**Schillinger & Lucke, 1989). Purified nisin was tested for the stability of activity against

Table I. Antibacterial titres of nisin against different sensitive bacteria as a function of pH CFS (cell free supernatant).

Strains	pH of CFS					
	3	4	5	6	7	8
Micrococcus luteus	1250	7500	7500	10000	10000	5040
Staphylococcus aureus	1000	4200	5040	5040	5040	3500
Bacillus cereus	500	1400	5000	5000	4800	1200

Table II. Amino acid composition of nisin produced using whey, MRS and standard nisin.

Amino acid	Conc.mg/g		
	Nisin (standard)	Nisin produced using MRS	Nisin produced using whey
Aspartic	3.7	6.6	8.8
Threonine	1.4	3.4	2.4
Serine	1.5	3.5	2.4
Glutamic	6.6	11.6	7.4
Proline	5.4	10.4	2.8
Glycine	1.7	6.0	1.6
Alanine	2.0	5.3	1.6
Cystine	0.2	0.3	1.2
Valine	1.8	3.9	1.8
Methionine	0.5	0.5	0.0
Isoleucine	1.8	2.9	2.0
Leucine	2.2	6.1	3.8
Tryosine	0.4	1.8	1.6
Phenylalanine	1.2	3.8	1.6
Histidine	0.7	1.7	1.4
Lysine	3.3	5.1	5.6
Arginine	1.3	3.8	0.6

Micrococcus luteus at different incubation temperatures from 10 to 100°C for 30 min. At 10 and 20°C incubation temperature, nisin activity achieved 9000 Au/ml and decreased to 7500 Au/mL at 30°C (Fig. 1). Stability of nisin activity was recorded at 40°C till 90°C attained 5040 Au mL^{-1}. Nisin was stable at high temperature and for long storage periods. The heat stability implies a very compact molecular structure. In addition, Lactococcin R proved to be stable to high temperatures at acidic pH values. However, biological activity was decreased at alkaline pH (Matsusaki *et al*., 1996 & Yildirim & Johnson, 1997).

Direct detection of nisin by SDS-PAGE. The rate of movement is mainly depends upon the charge and the mass and shape of the molecule. An increase in mass exerts a retarding effect. Also, opposing the movement of these proteins is the electroendosomatic flow from anode to cathode. Electrophoresis of nisin purified from *Lactococcus lactis* ATCC 11454 and *Lactococcus lactis* Fc2 exposed to different doses of gamma irradiation namely 0.0, 1, 1.5, 2, 2.5, 3 and 4 kGy was carried out. At 1.5 kGy dose, lane 5 showed over expression of nisin when compared with the other lanes (Fig. 2). A comparison with marker lane (no. 3), nisin had a molecular weight of 3.5 kDa. Kuipers *et al.* (1992) reported that nisin separation by SDS-PAGE uses certain amount of purified nisin and use Coomassie blue

Fig. 1. Antibacterial titres of nisin against Micrococcus luteus as a function of incubation temperatures.

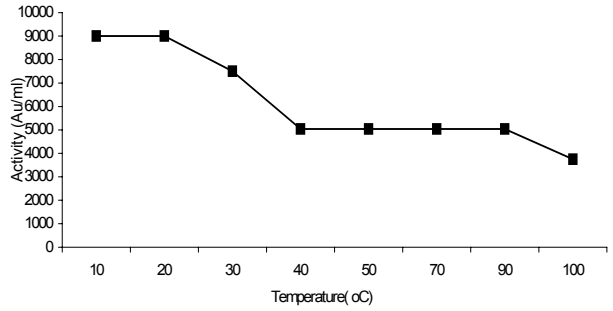
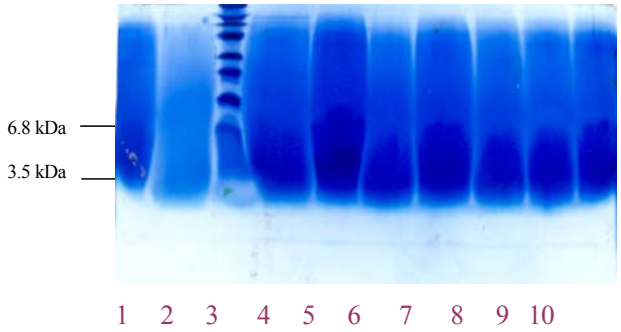


Fig. 2. Lane 1: nisin purified from *Lactococcus lactis* standard, lane 2: nisin purified from *Lactococcus lactis* (Fc2), lane 3: marker for molecular weight, lane 4: nisin standard, lane 5: nisin purified from Fc2 at 1.5 kGy, lane 6: nisin purified from Fc2 at 4 kGy, lane 7: nisin purified from Fc2 at 1 kGy, lane 8: nisin purified from Fc2 at 3 kGy, lane 9: nisin purified from Fc2 at 2 kGy, lane 10: nisin purified from Fc2 at 2.5 kGy.



have molecular weight of 3 kDa. Monomer could arise as reaction products from hydroxyl addition to dehydro groups or from intramolecular additions involving nucleophilic groups within the peptide. Multimers could arise from a variety of intermolecular reactions such as nucleophilic R groups from one molecule adding to dehydro groups of other nisin molecules (Hansen *et al.,* 1990). Nisin irradiated at 2 kGy started to decrease in the gene expression. Greaser *et al*. (1983) stated that samples of Lacticin F on SDS-PAGE gels could not be stained with Coomassie dye but could be stained with silver. Stained SDS-PAGE gels should moderately sized protein bands as well as a prominent band of 2.5 kDa (Klaenhammer & Muriana, 1990). However, in Fig. 3 electrophoresis of purified nisin after storage for two weeks gave the same result but nisin appeared in dimmer form means has a molecular weight of 7 kDa. Nisin on SDS-PAGE with Coomassie blue stain give one band, after storage for two weeks or more, produced several bands, some of which were diffused nisin as monomer very close to 3.5 kDa or 7 kDa as other references nisin have 2.5 kDa (Hansen *et al*., 1990 & Yildirim & Johnson, 1997).

Amino acids analysis for nisin. Nisin is an antibiotic produced by certain strains of *Streptococcus lactis*. It is a polypeptide containing three non-protein amino acids: Lanthionine, β-methyl lanthionine and dehydroalanine**.** It has a molecular weight of 3.5 kDa and is commonly found as stable, biologically active dimmers or tetrameters (Gross & Morell, 1971). The amino acids composition for nisin standard and nisin produced through MRS and Whey media indicated a rise in the concentration of almost amino acids in MRS medium (Table II). There was a great similarity in amino acids composition between nisin and other lanthionine containing peptides (Klaenhammer *et al*., 1991).

Amino acids produced through Whey medium were higher than that from standard nisin except in proline, glycine, alanine, methionine and arginine. Amino acids composition analysis revealed that nisin contained a majority of nonpolar amino acids and some lanthionine residues, cystine presence in three samples with smallest amount, this explain cystiene form lanthionine and β-methyl lanthionine (Klaenhammer *et al*.,1991).

Plasmid profile. The property of bacteriocin production seems to be hereditary characteristic of the bacterial cell, determined by cytoplasmic plasmids in general and genes located on chromosomes in particular (Klaenhammer *et al*., 1994). Gene responsible for nisin production relate to immunity gene and can be mediated on plasmid DNA in *Streptococcus lactis* (Nettles & Barefoot, 1993). Fig. 4 showed the plasmid profile of *Lactococcus lactis* Fc2 exposed to 0.0, 1.5 and 2 kGy dose level of gamma radiation in comparison with marker and *Lactococcus lactis* ATCC 11454 as well. Chromosomal DNA appeared as first band in lane 2, 3 and plasmid DNA bands had the same molecular weight of plasmid marker (promega). If

Fig. 3. Lane 1: Standard nisin, lane 2: nisin purified from *Lactococcus lactis* (Fc2) at 4kGy, lane 3: nisin purified from Fc2, lane 4: nisin purified from standard strain, lane 5: nisin purified from Fc2 at 3 kGy, lane 6: nisin purified from Fc2 at 2 kGy, lane 7: nisin purified from Fc2 at 2.5 kGy, lane 8: nisin purified from Fc2 at 1 kGy, lane 9: nisin purified from Fc2 at 1.5 kGy.

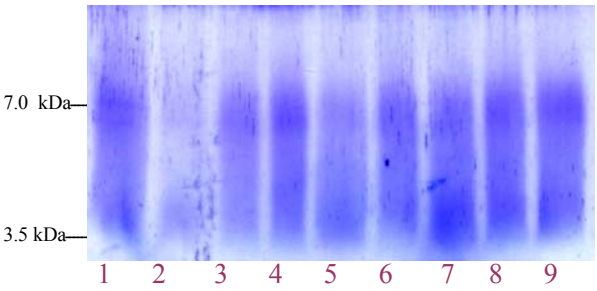
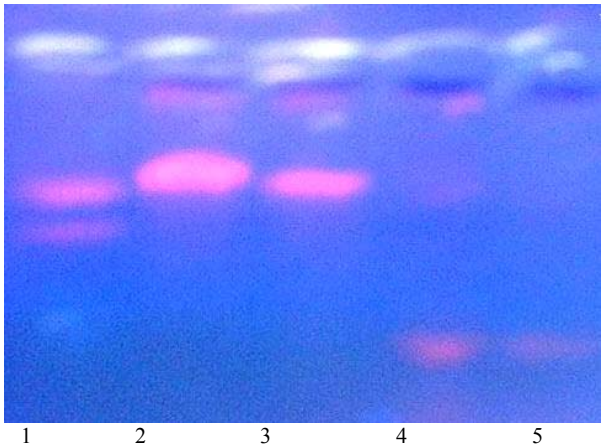


Fig. 4. Lane 1: Standard marker from promega, lane 2: plasmid isolated from *Lactococcus lactis* standard, lane 3: plasmid isolated from *Lactococcus lactis* (Fc2), lane 4: plasmid isolated from *Lactococcus lactis* (Fc2) at 1.5 kGy and lane 5: plasmid isolated from *Lactococcus lactis* (Fc2) at 2 kGy.



bacteriocin is plasmid encoded, one plasmid should be lost, but in case of bacteriocin is chromosomal encoded the plasmid profile of the wild type strain and its mutants should be similar, mean plasmid not lost (Enan *et al.,* 1994). Plasmid isolated at dose level 1.5 kGy, which gave high nisin production (Abdel Karem *et al*., 2005), was deficient due to exposing to gamma irradiation and had low molecular weight. The plasmid not lost probably due to cleavage occurred by radiation effect and at dose level of 2 kGy. DNA plasmid was more cleavage but not lost as shown in lane 5. Therefore, the determinants for nisin appeared to be chromosomal encoded. These findings corroborate the findings of Daeschel (1990) and Klaenhammer *et al*. (1994). Increase in nisin resistance for producer strain due to immunity genes lead to significant improvement in rate and level of nisin production (Kim *et al.,* 1997).

CONCLUSION

In conclusion factors like pH and temperature are important in stability of nisin activity. At neutral pH, nisin activity was higher and stable at high temperature for a long storage period. Molecular weight of nisin as determined by SDS-PAGE was 3.5 kDa at 1.5 kGy irradiation dose level and after storage for two weeks it had a molecular weight of 7 kDa. Nisin produced in Whey medium had higher activity than in MRS medium, although both had similar structure. Finally, nisin gene proved encoded by chromosome.

REFERENCES

Abdel Karem, H., H. Hussein, S. Badr and D. El–Hadedy, 2005. Factors resulting production of nisin by *Lactococcus lactis*. *New Egyptian J. Microbiol.*, 10: (in press).

Amiali, M.N., G. Lacroix and R.E. Simard, 1998. High nisin Z production by *Lactococcus lactis* UL719 in whey permeate with aeration. World. *J. Microbiol. Biotechnol.*, 14: 887–94

Chinachoti, N., T. Zaima, H. Matsusaki, K. Sonomoto and A. Ishisaki, 1997. Relationship between fermentative production and aeration conditions using *Lactococcus lactis* 10–1. *J. Fac. Agric. Kyushu Univ.*, 43: 421–36

Daeshel, M.A., 1990. Application of bacteriocins in food systems. *In:* Bills, D.D. and Kung, S. (eds.). *Biotechnology and Food Safety.* pp. 91–115. Butterworth. Heinemann, Boston.

Enan, G., M.A. El Sayed, A.A. El Essawy and J. Debevere, 1994. Production and characterization of a bacteriocin by *Lactobacillus plantarum* UGI. *In proceed. 8th Belg. FORUM Appl. Biotechnol.,* Brugge, Belgium, 28–30 Septem., 1994 pp. 1795–811

Greaser, M., R.L. Moss and G.G. Giulian, 1983. Improved methodology for analysis and quantification of proteins on one dimensional silver stained gels. *Anal. Biochem.*, 129: 227–87

Gross, E. and J.L. Morell, 1971. Peptide with alpha, beta unsaturated acids. In: E. Scoffons, (ed.). *Peptides 1969. Elsevier/North.* pp. 356–60. Holland Publishing Co. Amsterdam.

Hansen, J.N. and W. Liu, 1990. Some chemical and physical properties of nisin, a small protein antibiotic produced by *Lactococcus lactis*. *Appl. Environ. Microbiol.*, pp. 2551–8

Hansen, J.N., S. Banerjee and G.W. Buchman, 1990. Structure, organization, expression and evolution of the genes encoding the peptide precursors of nisin subtilin. *In:* Bills, D.D. and S. Kung, (ed.). *Biotechnology and Food Safety.* pp. 75–89. Butterworth–Heinemann, Boston.

Hurst, A., 1981. *Nisin. Adv. Appl. Microbiol.*, 27: 85–122

Hurst, A. and D.G. Hoover, 1983. Nisin. *In:* Davidson, P.M. and A.L. Branen, (eds.) *Amimicrobials in Foods,* 2nd pp. 369–94 New York, Basel, HongKong: Marcel Dekker.

Jack, R.W., J.R. Tagg and B. Ray, 1994. Bacteriocins of Gram–positive bacteria, *Microbiol. Rev.*, 59: 171–200

Kim, W.S., R.J. Hall and N.W. Dumm, 1997. The effect of nisin concentration and nutrient depletion on nisin production of *Lactococcus Lactis*. 48: 449–53

Klaenhammer, T.R., 1984. A general method for plasmid isolation in *Lactobacilli*. *Curr. Microbiol.*, 23–8

Klaenhammer, T.R. and P.M. Muriana, 1990. Purification and partial characterization of lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. *Appl. Environ. Microbiol.*, 57: 114–21

Klaenhammer, T.R, M.J. Desmazeaud, P.M. Muriana and J.C. Piard, 1991. Purification and partial characterization of lacticin 481, a lanthionine–containing bacteriocin produced by *Lactococcus lactis* subsp lactis CNRZ 481. *Appl. Environ. Microbiol.*, 58: 279–84

Klaenhammer, T.R, C. Ahn, C. Fremaux and K. Milton, 1994. Molecular biology of bacteriocins produced by *Lactobacilus*. *In:* Hoover, D.G. and L.R. Steenson, (eds.). *Bacteriocins of Lactic Acid Bacteria.* Academic press. Inc., New York.

Kuipers, P.D., J.S. Roland, W.M. John and M.D. Willem, 1992. Properties of nisin Z and distribution of its gene, nisZ, in *Lactococcus lactis*. *Appl. Environ. Microbiol.*, 59: 213–8

Lewus, C.B. and T.J. Montville, 1991. Detecation of bacteriocins from lacticacid bacteria. *J. Microbiol. Methods,* 13: 145–50

Matsusaki, H., N. End, A. Sonomato and A. Ishizaki, 1996. Lantibiotic nisin Z fermentative production by *Lactococcus lactis* 10–1: relationship between production of the lantibiotic, lactate and cell growth. *Appl. Microbiol. Biotechnol.*, 45: 36–40

Nettles, C.G. and S.F. Barefoots, 1993. Biochemical and genetic characteristics of bacteriocins of food associated lactic acid bacteria. *J. Food. Prot.*, 56: 338–56

Pucci, M.J., E.R. Vedamuthu, B.S. Kunka and P.A. Vandenbergh, 1988. Inhibition of *Listeria monocytogenes* by using bacteriocin PA–1 by *Pediococcus acidilactici* PAC 1.0. *Appl. Environ. Microbiol.*, 54: 2349–53

Schillinger, U. and F.K. Lucke, 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. *Appl. Environ. Microbiol.*, 55: 1901–6

Tramer, J. and G.G. Fowler, 1964. Estimation of nisin in foods. *J. of Sci. and Food Agric.,* 8: 522–8

Winder, K. and O.B. Eggum, 1966. Protein by hydrolysis. Adescription of the method used at the department of animal physiology in Copenhagen. *Acta Griculture. Scandinavia,* 16: 125

Wolf, C.E. and W.R. Gibbons, 1995. Improved method for quantification of bacteriocin nisin. *J. Appl. Bacteriol.*, 80: 453–7

Yang, Z., 2000. Antimicrobial Compounds and extracellular polysaccharides produced by lactic acid bacteria: Structure and properties. Academic Dissertation, Department of Food technology, University of Helsinki. Helsinki. Finland.

Yildirim, Z. and M.G. Johnson, 1997. Detection and characterization of bacteriocin produced by *Lactococcus lactis* subsp *cremois* R isolated from radish. *Lett. Appl. Microbiol.*, 26: 297–304

Zamfir, M., R. Callewaert, P.C. Conea and L. De vuyst, 2000. Production kinetics of acidophilin 801, a bacteriocin produced by *Lactobacillus acidophilus* IBB 801. *FEMS Microbiol. Lett.*, 190: 305–8

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