African Journal of Biotechnology Vol. 2 (7), pp. 179–184, July 2003
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2003 Academic Journals

Full Length Research Paper

Influence of cultural conditions on the production of bacteriocin by *Lactobacillus brevis* OG1

S.T. Ogunbanwo*, A.I. Sanni, and A. A. Onilude

Department of Botany and Microbiology, University of Ibadan, Nigeria

Accepted 5 June 2003

Bacteriocin produced by *Lactobacillus brevis* OG1 has large spectrum of inhibition against pathogenic, food spoilage microorganisms and various Lactic acid bacteria employed as test strains. The bacteriocin inhibited *E coli* NCTC 10418 and *Enterococcus faecalis*, but did not inhibit *Candida albicans* ATCC 10231 and *Klebsiella* sp. UCH 15. The antibacterial activity appeared to be pronounced between early logarithmic and early stationary phase. Supplementation and/or replacement of nutrients demonstrated that larger quantities of bacteriocin could be produced by addition of yeast extracts (3.0%), NaCl (1.0-2.0%), glucose (1.0 %) and Tween 80 (0.5%), while addition of tri-ammonium citrate, sodium acetate, magnesium sulphate, manganese sulphate and potassium phosphate had no effect on production. Maximal activity in composed medium was achieved at initial pH of 5.5, and incubation period of 48h at 30-37^{0}C.

Key words: Bacteriocin, growth media, *Lactobacillus brevis* OG1, indicator organisms, antagonistic activity.

INTRODUCTION

Lactobacilli are important organisms recognized for their fermentative ability as well as their health and nutritional benefits (Gilliland, 1990). They produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin or bactericidal proteins during lactic fermentations (Lindgren and Dobrogosz, 1990). The antimicrobial properties of lactobacilli are of special interest in developing strongly competitive starter cultures for food fermentation. Lactobacilli exert strong antagonistic activity against many microorganisms, including food spoilage organisms and pathogens. Production of the primary metabolite, lactic acid and the resulting pH decrease is the main preserving factor in food fermentation. In addition, some strains may contribute to the preservation of fermented foods by producing other inhibitory substances, such as bacteriocins (Brink et al., 1994). Bacteriocins are antimicrobial proteinaceous compounds that are inhibitory towards sensitive strains and are produced by both Gram-positive and Gram-negative bacteria (Tagg et al., 1976).

Research on bacteriocins from lactic acid bacteria has expanded during the last decades, to include the use of bacteriocins or the producer organisms as natural food preservatives. However, the antibacterial properties of lactic acid bacteria (LAB) have not been fully explored for use as biopreservative (Reddy et al., 1984) due to limited production techniques.

Maximal bacteriocin production could be obtained by supplementing a culture medium with growth limiting factors, such as sugars, vitamins and nitrogen sources, by regulating pH or by choosing the best-adapted culture medium (Vignolo et al., 1995). In order to exploit the potential utilitarian benefit of the antimicrobial properties of *Lactobacillus brevis* OG1, the present study was undertaken to determine the optimum cultural conditions for greater bacteriocin yield in constituted growth media.

MATERIALS AND METHODS

Sample collection

White maize (Zea mays) grains used in this study were obtained from retail markets in South-Western Nigeria.

*Corresponding author: Fax: 234-02-8103043; 8103118; e-mail: topzybanwo@yahoo.com

Table1. Inhibition of various indicator organisms by bacteriocin produced *L. brevis* OG1.

Organism	Strain No	Origin	*L. brevis*
Bacillus cereus	ATCC 9634	Reference strain	+(8mm)
Bacillus stearothermophillus	NCIB 8222	"	+(6mm)
Bacillus subtilis	ATC 6633	"	+(7mm)
Micrococcus futeus	NCIB 196	"	+(10mm)
Staphylococcus aureus	ATCC 14458	"	+(5mm)
Staphylococcus aureus	NCTC 6571	"	+(6mm)
Staphylococcus epidermidis	NCTC 5413	"	+(8mm)
Staphylococcus faecalis	ATCC 19433	"	-
Staphylococcus pyogenes	ATCC 19615	"	-
Listeria denitrificans	ATCC 14870	"	+(10mm)
Listeria monocytogenes	587CHRL	"	+(9mm)
Candida albicans	ATCC 10231	"	-
Escherichia coli	NCTC 10418	"	+(6mm)
Escherichia coli	K12	"	+(8mm)
Enterococcus faecalis	EFI	"	+(12mm)
Aeromonas pobvia	AP 15534	"	-
Vibro cholerea	AP 23622	"	+(8mm)
Shigella flexneri	AP 23498	"	+(5mm)
Shigella dysentry	AP 22433	"	+(5mm)
Salmonella typhimurium	ATCC 13311	"	+(6mm)
Salmonella kentucky	AT1	Reference strain	+(9mm)
Klebsiella spp	UCH15	Sputum	-
Clostridium sporagenes	NCIB 532	Reference strain	+(8mm)
Serratia marcescens	UI5	Soil	-
Helicobacter pylori	NCTC11637	Reference strain	-
Streptococcus thermophilus	IW 4	Iru	-
Lactobacillus acidophilus	U1	Ugba	-
Lactobacillus brevis	OG1	Ogi	-
Lactobacillus plantarum	F1	Fufu	+(8mm)
Lactobacillus reuteri	PW1	Palm wine	+(6mm)
Lactobacillus delbrueckii	PT6	Pito	-
Leuconostoc mesentaroides	M8	Meat	+(5mm)

Key**:** No inhibition = –; Inhibition = +, Zone of inhibition = (mm)

Traditional preparation of ogi

The cereal grains (Zea mays) are cleaned and steeped in water for 2 days in earthenware pot (or any suitable container). The water is decanted and the grains wet-milled before sieving with muslin cloth or fine wire-mesh. The pomace is discarded and the starch suspension is allowed to sediment during which fermentation is carried out for 2-3 days by the natural flora of the grains (Odunfa and Adeyele, 1985).

Bacterial Strains and Cultures

The strain of *L. brevis* OG1 used in this study was isolated from ogi and characterized using the API 50 CH strips (API System Biomerieux SA, France). It was maintained as frozen stocks at –20^{0}C in Hogness freezing medium and propagated twice in MRS medium (Oxoid; Onipath Ltd, Basingstoke, Hampshire, England) before use. The bacterial strains used as indicator organisms are listed in Table 1. All the LAB indicators organisms were cultivated in MRS medium while *Enterococcus, Micrococcus, Bacillus, Listeria* and *Staphylococcus*, were cultivated in brain heart infusion medium (Oxoid, UK). *Clostridium* was cultivated on blood agar while *Salmonella* and *Shigella* were cultivated in Desoxycholate-Citrate agar (DCA) medium. Gram-negative bacteria were cultivated in tryptic soy broth medium (Oxoid), while *Candida* was cultivated in yeast extract agar.

Bacteriocin Assay

L. brevis OG1 was cultivated anaerobically (Oxoid Gas Generating Kit) in MRS broth (glucose, 0.25%; peptone 0.5%) for 72 hours at 30°C. The pH and growth pattern of the test isolates was determined. Extraction of bacteriocin was carried out using the method of Schillinger and Lucke (1989). Inhibitory activity from hydrogen peroxide was eliminated by the addition of 5mg/ml catalase (C-100 bovine liver, sigma) (Daba et al., 1991). The culture supernatant was purified to ensure that only bacteriocin

Table 2. Effect of nutrient components on bacteriocin production by *L. brevis* OG1.

Medium Constituents	%	Growth (580nm)	Final pH	Bacteriocin Activity (AU/ml)
MRS (Normal)	-	0.9±003	3.90±020	3200±0.00
MRS + Tryptone	0.0	0.4±0.11	4.02±0.18	1600±0.00
	1.0	0.9±0.02	4.05±0.12	3200±0.00
	2.0	1.1±0.05	4.06±0.17	800±0.00
	3.0	1.4±0.01	4.08±0.20	800±0.00
MRS + Yeast extract	0.0	0.6±0.03	4.03±0.10	800±0.00
	1.0	0.9±0.05	4.01±0.19	3200±0.00
	2.0	1.2±0.03	4.02±0.15	6400±0.00
	3.0	1.4±0.02	4.04±0.11	6400±0.00
MRS + Beef Extract	0.0	0.4±0.01	3.92±0.12	1600±0.00
	1.0	0.9±0.03	3.95±0.16	3200±0.00
	2.0	1.1±0.02	3.98±0.14	800±0.00
	3.0	1.3±0.03	4.00±0.12	200±0.00
MRS + NaCl	0.0	0.9±0.02	3.90±0.15	3200±0.00
	1.0	0.7±0.04	3.93±0.18	6400±0.00
	2.0	0.6±0.01	3.95±0.20	6400±0.00
	3.0	0.4±0.02	3.99±0.12	800±0.00
MRS + glucose	0.0	0.4±0.03	4.12±0.14	1600±0.00
	1.0	0.7±0.05	4.01±0.12	6400±0.00
	2.0	0.9±0.02	3.96±0.16	3200±0.00
	3.0	1.2±0.02	3.93±0.13	800±0.00
MRS + Tween 80	0.0	0.6±0.01	4.12±0.16	800±0.00
	0.1	0.9±0.02	3.90±0.12	3200±0.00
	0.5	0.7±0.01	3.86±0.11	6400±0.00
	1.0	0.4±0.01	3.83±0.14	1600±0.00
MRS+Tri-ammonium citrate	0.0	0.4±.0.03	4.06±0.18	3200±0.00
	0.1	0.7±0.01	4.07±0.15	3200±0.00
	0.2	0.9±0.01	4.09±0.12	3200 ±0.00
MRS+S0dium acetate	0.0	0.4±0.00	4.05±0.14	3200±0.00
	0.1	0.6±0.02	4.08±0.12	3200±0.00
	0.2	0.7±0.01	4.10±0.17	3200±0.00
	0.3	0.9±0.01	4.13±0.15	3200±0.00
MRS+$MgSo_4.7H_20$	0.0	0.6±0.02	3.91±0.12	3200±0.00
	0.1	0.9±0.01	4.00±0.11	3200±0.00
	0.2	0.7±0.01	3.96±0.16	3200±0.00
	0.3	0.6±0.03	3.94±0.11	3200±0.00
	0.4	0.5±0.01	3.91±0.14	3200±0.00
	0.5	0.5±0.01	3.90±0.11	3200±0.00
MRS+$MnS0_4$	0.0	0.8±0.00	3.94±0.15	3200±0.00
	0.1	0.6±0.01	3.96±0.12	3200±0.00
	0.2	0.5±0.01	3.97±0.14	3200±0.00
	0.3	0.4±0.02	3.99±0.11	3200±0.00
MRS+K_2HP0_4	0.0	0.6±0.01	3.92±0.14	3200±0.00
	0.1	0.8±0.01	3.96±0.11	3200±0.00
	0.2	0.9±0.01	3.99±0.12	3200±0.00

(with the exception of other antimicrobial compounds such as lactic acid, hydrogen peroxide, diacetyl, ions etc) was assayed according to the method of Jimenez-Diaz et al. (1993). Bacteriocin was detected in the fraction at every stages of the purification. Antagonistic activity against indicator organisms was determined using a well diffusion assay (Schillinger and Lucke, 1989). The antimicrobial activity of the bacteriocin is defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and is expressed in activity units per ml (AU/ml).

Influence of medium component on the production of bacteriocin

The effect of medium ingredients on bacteriocin production was evaluated using composed media. The supplements studied were tryptone (0.0 – 3.0%), yeast extract (0.0 – 3.0%), beef extract (0.0 – 3.0%), triammonium citrate (0.0 – 0.2%) sodium acetate (0.0 – 0.5%), $MgSO_4{\cdot}7H_2O$ (0.0 – 0.5%), $MnSO_4{\cdot}4H_2O$ (0.0 – 0.1%), K_2HPO_4 (0.0 – 0.2%), NaCl (0.0 – 0.3%), glucose (0.0 – 3.0%) and tween 80 (0.0 – 1.0%).

Influence of growth conditions on the production of bacteriocin

The effect of incubation temperature and time on production of bacteriocin was carried out. Three portions of composed media were inoculated (1% v/v) with an overnight culture of bacteriocin producing organism; incubated at 25, 30, 37 45 and 55^{0}C for 48h and the absorbance values (580nm), pH and bacteriocin activities of cultures were determined.

To determine the effect of initial pH on production of bacteriocin, 100ml of composed media were adjusted to initial pH values of 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5, using 5 mMol^{-1} hydrochloric acid or 5mMol1^{-1} NaOH. Each medium was inoculated (1% v/v) with an overnight culture of bacteriocin producing organism and incubated at 30^{0}C for 48 h. Absorbance values (580 nm), pH and bacteriocin activities were determined (Yildirim and Johnson, 1998).

The effect of incubation period was studied in the same manner as described for pH. Active cultures of producer organism (1% v/v) were inoculated into 100ml aliquots of sterile composed media in Erlenmeyer conical flasks. Inoculated flasks were incubated at 37^{0}C for periods of 12, 24, 36, 48, 60 and 72 h. Individual flasks were kept for each incubation period. At the end of each incubation period, bacteriocin activity, pH and absorbance values (580 nm) were determined (Balasubramanyam and Varadaraj, 1998).

RESULTS AND DISCUSSION

The present study was primarily aimed at determining cultural conditions for obtaining better and stable bacteriocin production. *L. brevis* OG1 was able to produce bacteriocin, which had a wide inhibitory spectrum towards both Gram-negative and Gram-positive food spoilage and pathogenic bacteria. It inhibited 21 out of 32 indicator strains with the largest zone of inhibition (12mm) being against *Enterococcus faecalis* EFI. The bacteriocin from *L. brevis* OG1 (producer organism) had no inhibitory effect on the organism itself (Table 1). In a mixed fermentation environment, production of bacteriocins may prove advantageous for a producer organism to dominate the microbial population (Graciela, 1995).

The influence of culture medium components on the production of bacteriocins was investigated using *E. faecalis* EFI as indicator organism. Results show that bacteriocin was produced when nutrients were available for metabolic activity. Larger amounts of the bacteriocin were synthesized only when the medium was supplemented with glucose (1.0%), Tween 80 (0.5%), yeast extract (2-3.0%) and NaCl (1-2.0%), while addition of tri-ammonium citrate, sodium acetate, magnesium sulphate, manganese sulphate and potassium phosphate had no effect on bacteriocin production (Table 2). Thus variation in the concentration of constituents/ supplementation of cultivation media might have an influence on the amount of bacteriocin produced by microorganisms. Similar observations have been made previously. Daba et al. (1993) obtained similar results in the production of mensenterocin 5. Biswas et al. (1991) compared the production of pediocin ACH by *Pediococcus acidilactici* H cultivated in TGE broth, MRS broth and several modifications of it. TGE broth containing varied concentration (0-2%) of trypticase, glucose and yeast extract, produced maximum pediocin at the 1% level. When cultivated in normal MRS broth, 15% less pediocin was obtained compared to the yield in TGE broth. Sanni et al. (1999) also reported that highest bacteriocin activity was obtained when glucose and peptone were varied to 0.25% and 0.5% in the constituted MRS broth, while bacteriocin activity was not detected at 2% glucose and peptone level. Modification of nutrients of cultivation media should be considered for maximal production of bacteriocin that has potential use as a food biopreservative (Biswas et al., 1991). The question of production cost is an important issue to be taken into account when large-scale production of the bacteriocin for use as a food preservative is considered. Our results also show that bacteriocin could be produced in a relatively inexpensive medium.

Bacteriocin production by the test isolate displayed secondary metabolite kinetics. For example, bacteriocin was produced during the pre-and early exponential growth phases and reached a maximum level at late stationary phase. Growth beyond the stationary phase resulted in a decrease in bacteriocin activity (Figure 1a). This decrease could be due to the activity of extracellular endogenous proteinases induced during this growth phase. Some reports indicate that bacteriocins are produced throughout the experimental growth phase and not solely during late logarithmic or early stationary phase (Joerger and Klaenhammer, 1986; Piard et al., 1990).

The effect of incubation temperature, incubation period and initial pH of medium on production of bacteriocin was also investigated. The use of constituted medium at 30^{0}C incubation temperature, initial pH 5.5 and for 48 to 60 hours fostered the best production of bacteriocin by *Lactobacillus brevis* OG1 (Figures 1a - c).

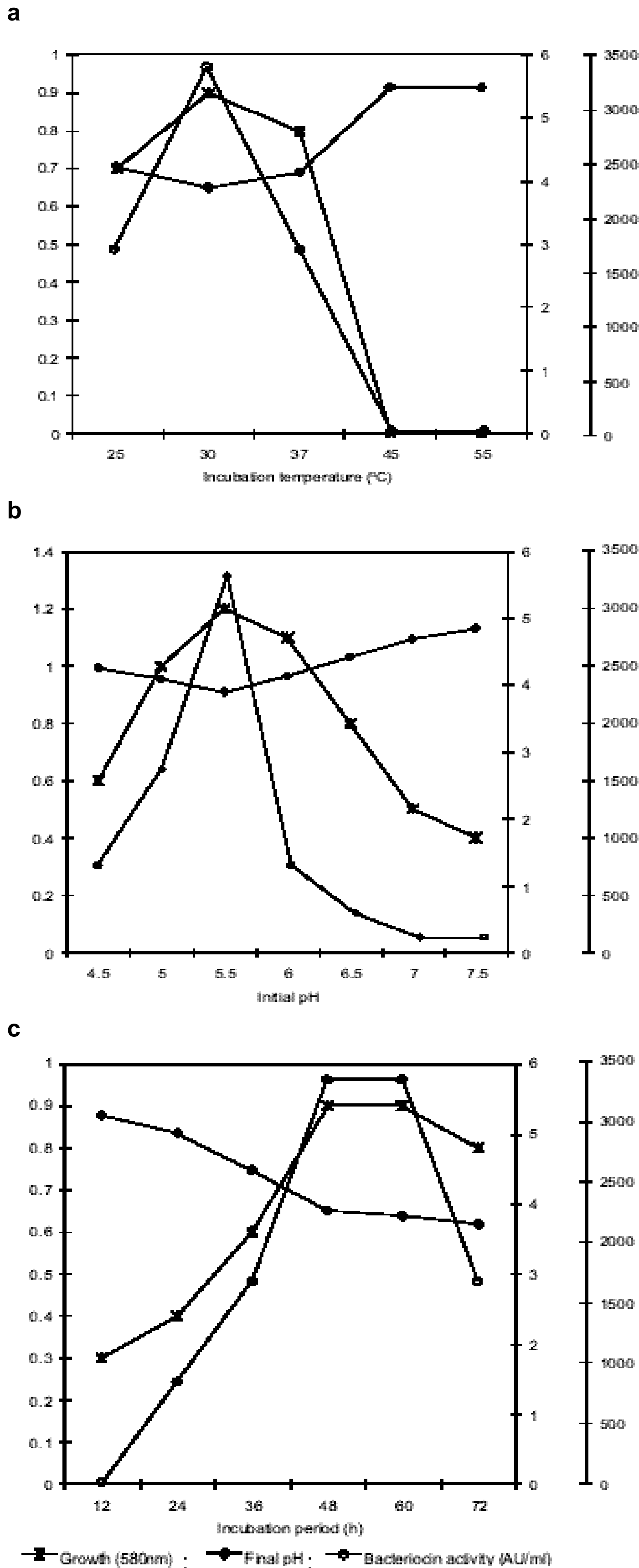

Figure 1. Effect of Incubation temperature, pH and incubation period on the activity of bacteriocin produced by *L. brevis* OG1.

ACKNOWLEDGEMENTS

The authors thank Dr S. I. Smith of Genetics Laboratory, Nigeria Institute of Medical Research, Yaba, Lagos, Nigeria for technical assistance.

REFERENCES

Balasubramanyam BV, Varadaraj MC (1998). Cultural conditions for the production of bacteriocin by a native isolate of *L. delbrueck*, ssp. Bulgaricus CFR 2028 in milk medium. J. Appl. Microbiol. 84: 97 – 102.

Biswas SR, Ray P, Johnson MC, Ray B (1991). Influence of growth conditions on the production of a bacteriocin, pediocin ACH, by pediococcus acidilactic H. Appl. Environ. Microbiol. 57: 1265 – 1267.

Brink ten B, Minekns M, Vander Vossen JMBM, Leer RJ, Huis in't Veld JHJ (1994). Antimcrobial activity of lactobacilli. J. Appl. Bacteriol. 77: 140 – 148.

Daba H, Lacroix C, Hang J, Simard RE, (1993). Influence of growth conditions on production and activity of mesenteric 5 by a strain of *Leuconostoc mesenteries*. Appl. Microbiol. Biotechnol. 39: 166 – 173.

Daba H, Pandian S, Gosselin JF, Simard RE, Huang J, Lacroix C (1991) Detection and activity of bacteriocin produced by *Leuconostoc mesenteriodes*. Appl. Environ. Microbiol. 57: 3450-3455.

Fricourt BV, Barefoot SF, Testin RF, Hayasaka SS (1994). Detection and activity of Plantaricin F an antibacteria substance from Lactobacillus plantarum BFOO1 isolated from processed channel catfish. J. Food. Prot. 37 (8): 698-708.

Gilliland SE (1990). Health and Nutritional benefits from lactic acid bacteria. FEMS Microbial. Rev. 87: 175 – 178.

Graciela M, Vignolo M, de Kairuz M, Aida AP, de Ruiz H, Oilver G (1995). Influence of growth conditions on the production of lactocin 705, a bacteriocin produced by *L. casei* CRL 705, J. Appl. Bacteriol. 78: 5 – 10.

Jimenez-Diaz R, Rios-Sanchez RM, Desmazeaud M, Ruiz-Barba JL, Piard JC (1993). Plantaricins S and T. Two New bacteriocins produced by *L. plantarum* LPCO10 isolated from a green olive fermentation. Appl. Environ. Microbiol. 59: 1416-1424.

Joerger MC, Klaenhammer TR (1986). Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. J. Bacteriol. 167: 439 – 446.

Lindgren SW, Dobrogosz WJ (1990). Antagonistic activities of lactic acid bacteria in food and feed fermentation. FEMS Microbiol. Rev.87: 149-164.

Odunfa SA, Adeyele SJ (1985). Microbiological changes during the traditional production of ogi-baba, a West African fermented Sorghum gruel. J. Cereal Sci. 3:173-180.

Piard JC, Delorme F, Giraffa G, Commissaire J, Desmazeaud M (1990). Evidence for a bacteriocin produced by *Lactococcus lactis* CNRZ 481. J. Neth. Milk Dairy 44: 143 – 158.

Reddy GC, Shahani KM, Friend BA, Chandan RC (1984). Natural antibiotic activity of *L. acidophilus* and bulgaricus, production and partial purification of Bulgaricus cultured. J. Dairy Products 8: 15 – 19.

Sanni AI, Onilude AA, Ogunbanwo ST, Simth SI (1999). Antagonistic activity of bacteriocin produced by Lactobacillus species from ogi, an indigenous fermented food. J. Basic Microbiol. 39: 189-195.

Schillinger U, Lucke FK (1989). Antimicrobial activity of Lactobacillus sake isolated from meat. Appl. Environ. Microbiol. 55:1901-1906.

Tagg JR, Dajani AS, Wannamaker LW (1976). Bacteriocins of grampositive bacteria. Bacteriol. Rev. 40: 722 – 756.

Vignolo GM, de Kairuz MN, de Ruiz Holgado AAP, Oliver G (1995). Influence of growth conditions on the production of lactocin 705, a bacteriocin produced by *Lactobacillus casei* CRL 705.. J. Appl. Bacteriol. 78: 5-1.

Yildirim Z, Johnson MG (1998). Detection and Characterization of a bacteriocin produced by *Lactococcus Lactis subsp.* Cremoris R. isolated from radish. Lett. Appl. Microbiol. 26: 297 – 304.