JOURNAL OF CULTURE COLLECTIONS
Volume 5, 2006-2007, pp. 58-63

ISOLATION OF BACTERIOCINOGENIC LACTIC ACID BACTERIA FROM RAT INTESTINE

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Summary

Lactic acid bacteria were isolated from rat intestinal contents and screened for bacteriocin production. Out of twenty-three isolates, eight were found to produce bacteriocins. The morphological and biochemical properties of these isolates indicated that they belong to the genus Enterococcus. The bacteriocins were extracted and partially purified by Gel Permeation Chromatography (GPC) using G-25, and the antimicrobial spectrum of these bacteriocins was studied against indicator organisms, including Lactococcus diacetylactis, Staphylococcus aureus, Escherichia coli and Pseudomonas sp.

Key words: *lactic acid bacteria, bacteriocins, Enterococcus, intestine.*

Introduction

Lactic acid bacteria (LAB) are industrially important organisms because of their fermentative ability as well as health and nutritional benefits. Moreover, they are generally regarded as safe for incorporation into food products [2]. The ability of lactic acid bacteria to inhibit the growth of various Gram positive and/or Gram negative bacteria is well known and attributed to the production of organic acids such as lactic acid and acetic acid [4], hydrogen peroxide, bacteriocins, bacteriocin like substances and possibly biosurfactants [14]. Bacteriocins are ribosomally synthesized, extracellular peptides/proteins produced by bacteria, exhibiting bacteriostatic or bacteriocidal activity against closely related bacteria. These molecules are produced by bacteria belonging to several genera, however, only some of them have been extensively studied. Bacteriocins produced by lactic acid bacteria have received considerable attention in recent years, because of their possible use as biopreservatives in food processing industry with resultant reduction in the use of chemical preservatives [15]. Another advantage of bacteriocins for use as a biopreservatives is that they can be easily digested in the human gastro-intestinal tract [11]. Earlier, several researchers have isolated LAB from dairy and meat products, fermented vegetables and mucosal surfaces of animals [5, 8, 16]. Bacteriocin producing species have now been identified among all the genera that comprise the LAB including *Lactobacillus, Pediococcus* and *Carnobacterium* [6, 12] as well as several *Enterococcus* species [1, 9].

In the present study LAB have been isolated and characterized from the intestinal contents of Wistar albino rat and screened for bacteriocin production.

Materials and Methods

Chemicals. General chemicals and solvents of analytical grade were procured from SRL, India and S.D Fine Chemicals, India, while the molecular weight markers and bacteriological media were obtained from Sigma, USA and HiMedia, India, respectively.

Bacterial strains. The indicator organisms viz, *Staphylococcus aureus* and *Lactococcus diacetylactis* were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India, while *Escherichia coli* and *Pseudomonas* sp. were isolated and identified at Defence Food Research Laboratory, Mysore, India.

Isolation of lactic acid bacteria. LAB were isolated from intestinal contents of 10 months old Wistar rat. The rat was fed with the Nutrilab-rodent feed procured from Tetragon Chemie Pvt Ltd, Bangalore, India. One gram of intestinal content was suspended in 9 ml of 0.85 % sterile saline. The solution was further diluted with saline and 0.1 ml of the solution was spread on MRS agar and incubated in anaerobic jar at 37 ^{o}C for 2 days. The colonies were randomly picked and transferred to MRS broth. Further purification was achieved through streaking on MRS agar to pick up pure colonies. The cultures were further stored in MRS agar overlaid with 50 % glycerol at minus 20 ^{o}C. A total of twenty-three isolates were tested for their ability to produce bacteriocins.

Screening of LAB for antimicrobial activity. All the isolates maintained in frozen stocks were propagated twice in MRS broth and used for further study. For screening the isolates for bacteriocin production all isolates were grown in MRS broth and incubated at 37 ^{o}C for 60 h. The cell free supernatants were adjusted to pH 5.0 with 2N NaOH and concentrated to one tenth of the original volume by rotary flash evaporator. These samples were filter sterilized by passing through 0.22 μm membrane filter (Millipore, India) and evaluated for antimicrobial activity by agar well diffusion method [14] against *S. aureus* and *L. diacetylactis*.

Characterization of LAB isolates. Fresh (12-18 h) cultures of bacteriocin producing isolates were Gram stained and examined microscopically. Catalase activity was tested by adding few drops of 3 % hydrogen peroxide to a test tube containing 24 h old culture of each isolate.

Growth was assessed in MRS broth at 15, 37 and 45 ^{o}C and at pH 4.4, 7.0, 8.6 and 9.6 with incubation at 37 ^{o}C. Salt tolerance was tested with 6.5, 10 and 15 % (w/v) NaCl in MRS broth. Production of acid and CO_2 was tested in MRS broth containing inverted Durham's tube with citrate omission in the medium [13]. Ammonia production test was conducted in MRS broth in the absence of glucose and meat extract, containing 0.3 % arginine and 0.2 % sodium citrate in place of ammonium citrate. The production of ammonia was confirmed using Nessler's reagent. Homo- and heterofermentative tests were performed according to the method of Zuniga et al [18]. Ability to ferment various sugars was examined using HiCarbohydrate^{TM} kit Hi-Media, India.

Cell-wall protein extraction and analysis. LAB isolates were grown in MRS broth. The cell mass was harvested and resuspended in 3 ml of distilled water (A_{600} ~ 2) and centrifuged. Cell wall proteins were extracted from the final cell pellet with 0.5 ml of 0.01 M Tris-HCl, 0.01M EDTA, 0.01M NaCl, 2 % SDS, pH 8.0 at 100 ^{o}C for 5 min [3]. After treatment the supernatants were centrifuged at 11,600 g for 10 min and analyzed by Tris-Glycine SDS-PAGE [9] with 5 % stacking and 12 % separating gel.

Extraction and partial purification of bacteriocins. Cell adsorption-desorption method [7] was employed for bacteriocin extraction from 72 h old cultures. Bacteriocins were adsorbed on to the cells at pH 6.5 and desorption was carried out at pH 2.0-2.5, in the presence of 0.1 N NaCl. The contents were further concentrated by rotary flash evaporator followed by adjusting pH to 4.5. The concentrated samples were further purified by passing through a Sephadex G-25 column and eluted with ammonium acetate buffer (0.05M, pH 4.5). Various fractions around shoulder, peak and valley were pooled separately, further concentrated and checked for the inhibitory activity against *S. aureus*. Subsequently, these active fractions were used to verify the antimicrobial activity against various indicator organisms.

Results and Discussion

Lactic acid bacteria are known to produce a large variety of bacteriocins and some of them are extensively used to extend the shelf-life of food products by inhibiting the growth of food born pathogens and spoilage microorganisms. In this study we have attempted to isolate LAB from intestinal contents of Wistar albino rat for production of potential bacteriocins. Twenty-three bacterial isolates were screened for bacteriocin production, out of which only eight produced bacteriocins active against *S. aureus, E. coli* and *L. diacetylactis*. Further, these isolates were characterized for their biochemical and physiological properties.

The morphological and biochemical properties of these isolates have been shown in Tables 1 and 2. All the isolates were Gram-positive cocci with slight variation in the cell arrangement. Isolates R1, R6, R7, R11 and R19 were found to exist in pairs and small chains while isolates R20 and R21 were short chains and isolate R12 existed in pairs only. These isolates were catalase negative and did not produce CO_2 from glucose and were found to be homofermentative. All the isolates were able to produce ammonia from arginine and acid from glucose. The isolates R1, R7, R11, R19 and R20 were able to grow even at 15 ^oC and 45 ^oC with maximum growth at 37 ^oC. In case of R6, R12 and R21 no growth was noticed at 15 ^oC. The growth of all isolates was found to be weak at pH 4.4 as well as at 6.5 % salt concentration. All the isolates grew luxuriously at pH values 8.6 as well as 9.6 and there was no growth at 4 ^oC and 10 % salt concentration in MRS medium. All the isolates produced acid from cellobiose, fructose, galactose, glucose, glycerol, lactose, mannose, maltose, mannitol, ribose, salicin and hydrolyzed the esculin. Isolates R1, R6, R11, R19, R20 and R 21 were able to ferment melizitose. In contrast, isolates R7 and R12 were able to ferment mellibiose.

On the basis of morphological and biochemical characteristics the isolates were assigned to the genus *Enterococcus*. Fermentation of melizitose and inability to ferment mellibiose helped in distinguishing between *E. faecalis* and *E. faecium* [10]. Based on the above tests, isolates R1, R6, R11, R19, R20 and R21 were identified as *E. faecalis* while isolates R7 and R12 were grouped into *E. faecium*, however, it was clear that they were different strains as they could be distinguished based on sugar fermentation pattern.

Table 1. Morphological, physiological and biochemical characteristics of bacteriocinogenic rat isolates.

Tests		Isolates							
		R1	R6	R7	R11	R12	R19	R20	R21
Morphology		Cocci Pairs & chains	Cocci Pairs & chains	Cocci Pairs & chains	Cocci Pairs & chains	Cocci Pairs	Cocci Pairs & chains	Cocci Small chains	Cocci Small chains
Growth at temperature (^oC)	4	-	-	-	-	-	-	-	-
	15	+	-	+	+	-	+	+	-
	37	++	++	++	++	++	++	++	++
	45	+	+	+	+	+	+	+	+
Growth at pH	4.4	w	w	w	w	w	w	w	W
	8.6	++	++	++	++	++	++	++	++
	9.6	++	++	++	++	++	++	++	++
Growth at NaCl (%)	6.5	w	w	w	w	w	w	w	w
	10	-	-	-	-	-	-	-	-
CO_2 from Glc		-	-	-	-	-	-	-	-
Acid from Glc		+	+	+	+	+	+	+	+
NH_3 from Arg		+	+	+	+	+	+	+	+
HHD Medium		Ho	Ho	Ho	Ho	Ho	Ho	Ho	Ho
Catalase		-	-	-	-	-	-	-	-

Legend: growth (+), no growth (-), luxurious growth (++), weak growth (w), arginine (Arg), glucose (Glc), homofermentative (Ho).

Table 2. Carbohydrate utilization pattern of bacteriocinogenic rat isolates.

Carbohydrates	Isolates							
	R1	R6	R7	R11	R12	R19	R20	R21
Adonitol	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-
Cellobiose	+	+	+	+	+	+	+	+
Citrate	-	-	-	-	-	-	-	
Dextrose	+	-	+	-	+	-	+	-
Dulcitol	-	-	-	-	-	-	-	-
Esculin	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+
Malonate	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+
Melezitose	+	+	-	+	-	+	+	+
Melibiose	-	-	+	-	+	-	-	-
ONPG	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-
Ribose	+	+	+	+	+	+	+	+
Rhamnose	-	-	-	-	-	-	-	-
Salicin	+	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	+	-	+	-
Sorbose	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-

Legend: growth (+), no growth (-).

Cell-wall protein extraction and analysis

Cell wall protein profile for eight bacteriocin producing LAB isolates has been depicted in Fig. 1. The electrophoregram revealed great similarity among the eight isolates indicating that all of them belong to the same genus. In all isolates high molecular weight proteins in the range of 36–66 kDa were more prominent than others.

Extraction and partial purification of bacteriocins

Bacteriocins obtained from cell adsorption-desorption technique were subjected to Sephadex G-25 column chromatography and recovered in partially purified form in shoulder and peak regions of the elution profile as shown in Fig. 2. Active fractions obtained with all the strains were concentrated and equal quantity of protein was used to check antimicrobial activity against selected indicator organisms. The bacteriocins from R19 and R20 isolates were found to be most potent against *S. aureus* while R7 bacteriocin had the least inhibitory activity. All the strains were found to inhibit *Pseudomonas* sp., *E. coli* and *L. diacetylactis* to varying extents. Further studies are in progress for the purification and characterization of these bacteriocins from the isolates in order to ascertain their possible use against food borne pathogens, which are not inhibited by the bacteriocins reported till date.

Acknowledgements. The authors are thankful to Dr. A. S. Bawa, Director, Defence Food Research Laboratory, Mysore, for providing the necessary facilities, constant guidance and encouragement.

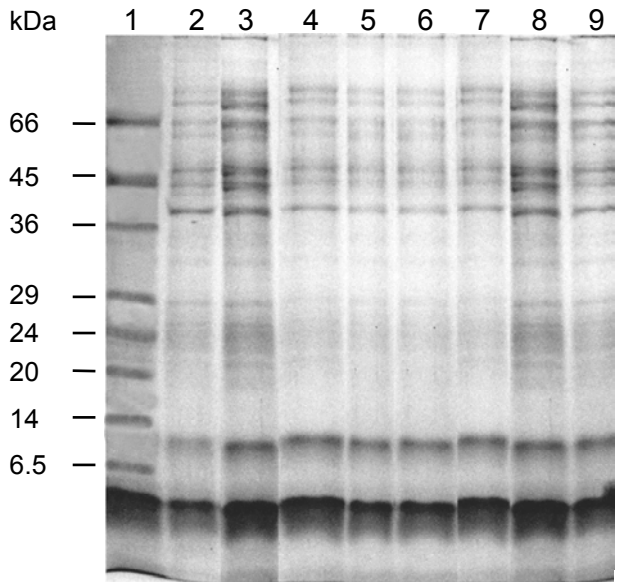

Fig. 1. Cell wall protein profile of rat intestinal isolates. Lanes; MW markers (1), R1 (2), R6 (3), R7 (4), R11 (5), R12 (6), R19 (7), R20 (8), R21 (9).

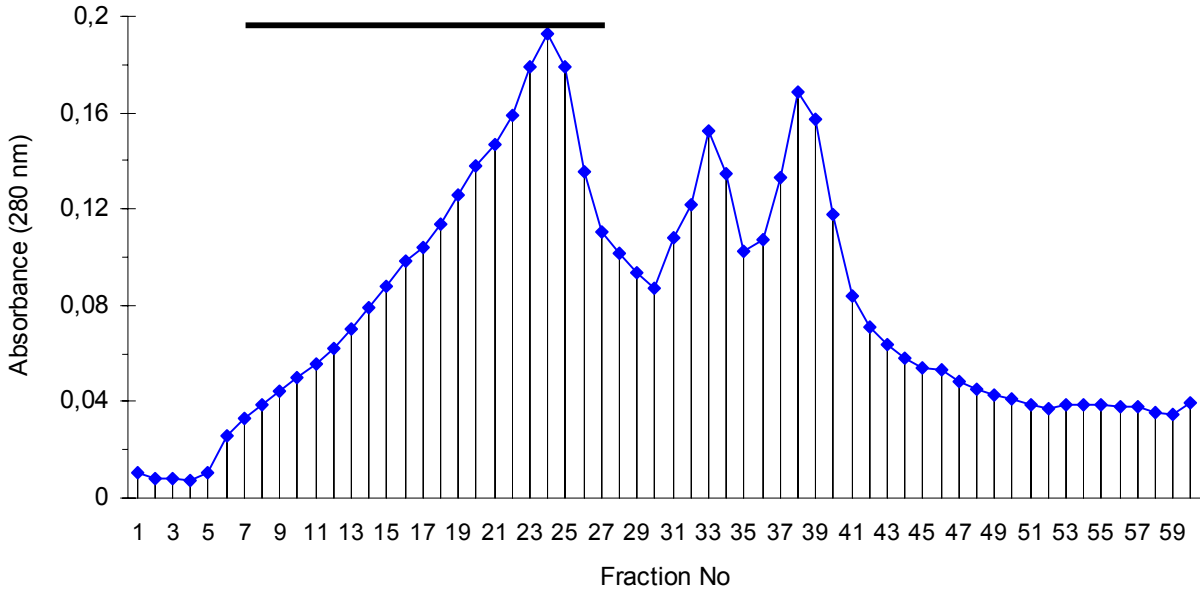

Fig. 2. Elution profile of bacteriocins on Sephadex G-25 column.
Absorbance at 280nm (♦). Active fractions 7-27 indicated by the bar (▬).

References

1. Arihara, K., S. Ogihara, J. Sakata, M. Itoh, Y. Kondo, 1991. *Lett. Appl. Microbiol.*, **13,**190-192.
2. Gasser, F., 1994. *Bull. Ins. Pasteur.*, **92**, 45-47.
3. Gatti, M., E. Fornasari, E. Neviani, 1997. *Appl Microbiol.*, **25**, 345-348.
4. Gilliland, S. E., M. L. Speek, 1977. *J. Food Protection.*, **40**, 820-823.
5. Jamuna, M., K. Jeevaratnam, 2004. *J. Appl Microbiol.*,**50**, 79-90.
6. Klaenhammer, T. R., 1998. *Biochemie*, **70**, 337-349.
7. Laemmli, U. K., 1970. *Nature*, **227**, 680-685.
8. Lindgren, S. W., W. J. Dobrogosz, 1990, *Microbiol. Rev.*, **87**, 149-164.

9. McKay, A. M., 1990. *Lett. Appl. Microbiol*., **11**, 15-17.
10. Mundt, O. J., 1986. Enterococci. In: *Bergey's manual of systematic bacteriology*, P. H. A, Sneath, N. S. Mair, M. E. Sharpe, J. G. Holt (Eds), Vol. 2, Baltimore: The Williams & Wilkins Co., 1063-1065.
11. Nurliza, B., K. Jan, B. L. John, 1998. *Appl. Environ. Microbiol*., **64**, 4842-4845.
12. Piard, J. C., M. Desmazeaud, 1992. *Lait.*, **72**, 113-142.
13. Schillinger, U., F. K. Lucke, 1987. *Food Microbiol*., **4**, 199-208.
14. Tagg, J. R., A. R. McGiven, 1971. *Appl Microbiol.*, **21**,125.
15. van Reenen, C. A., L. M. T. Dicks, M. L. Chikindas, 1998. *J. Appl. Microbiol*., **84**, 1131-1137.
16. Vijai, P., M. Jamuna, K. Jeevaratnam. 2004-2005. *J. Cult. Collections* (Sofia), **4**, 53-60.
17. Zuniga, M., I. Pardo, S. Ferrer, 1993. *Int J. Food Microbiol.*, **18**, 37-42.

ИЗОЛИРАНЕ НА БАКТЕРИОЦИНОГЕННИ МЛЕЧНОКИСЕЛИ БАКТЕРИИ ОТ ЧРЕВНИЯ ТРАКТ НА ПЛЪХ

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Резюме

Изолирани са млечнокисели бактерии от чревното съдържимо на плъх и са скринирани за продукция на бактериоцини. Установено е, че от 23 изолата 8 произвеждат бактериоцини. Морфологичните и биохимичните свойства на тези изолати показват, че принадлежат към род Enterococcus. Бактериоцините са екстрахирани и частично пречистени чрез гелна хроматография (GPC) при използване на Сефадекс G-25. Изследван е антимикробният им спектър срещу тест-организмите Lactococcus diacetylactis, Staphylococcus aureus, Escherichia coli и Pseudomonas sp.