Purification and characterization of enterocin FH 99 produced by a faecal isolate *Enterococcus faecium* FH 99

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Abstract *Enterococcus faecium* FH 99 was isolated from human faeces and selected because of its broad spectrum of inhibitory activity against several Gram-positive food-borne spoilage and pathogenic bacteria. *Ent. faecium* FH 99 accumulates enterocin in large number in early stationary phase of the growth. The enterocin FH 99 was stable over a wide pH range (2–10) and recovered activity even after treatment at high temperatures (10 min at 100°C). The enterocin was subjected to different purification techniques viz., gel filtration, cation exchange chromatography and reverse-phase high-performance liquid chromatography. The activity was eluted as one individual active fraction. SDS-PAGE revealed a molecular weight of less than 6.5 kDa. Studies carried out to identify the genetic determinants for bacteriocin production showed that this trait may be plasmid encoded as loss in both of the plasmids (size > chromosomal DNA) led to loss in bacteriocin production by *Ent. faecium* FH 99. *Ent. faecium* strain FH 99 is a newly discovered high bacteriocin producer with Activity Units 1.8×10^5 AU ml⁻¹ and its characteristics indicate that it may have strong potential for application as a protective agent against pathogens and spoilage bacteria in foods.

Keywords Bacteriocin · Enterocin · *Ent. faecium* · Food spoilage bacteria

Introduction

Studies on bacteriocins produced by lactic acid bacteria (LAB), which are bactericidal peptides and proteins [1], have attracted increasing interest in recent years because of their potential use as biopreservatives in the food industry to eliminate spoilage and food borne pathogenic bacteria [2]. Enterococci, the Gram positive bacteria, which fit under general definition of lactic acid bacteria, are often isolated from dairy systems as desirable microbiota and are believed to play an important role in cheese ripening due to their high lipolytic, proteolytic and flavour producing activities.

Investigations have resulted in the recognition that production of antilisterial bacteriocins is a common characteristic within this group of bacteria. Enterocins, the antibacterial peptides produced by *Enterococcus* species are small, hydrophobic, and thermostable with activity over wide range of pH. These generally belong to class II [3]. Enterocins are active against many Gram-positive bacteria encompassing undesirable and pathogenic microbes, such as *Clostridia*, *Bacillus*, *Staphylococcus* and *Listeria*, as well as some Gram-negative bacteria [4]. This fact stimulates the special interest in enterocins as potential food preservatives. Although enterocins have yet not been allowed to be used as such in food products, nevertheless bacteriocinogenic enterococci can still be used as starter and/ or adjunct culture for the *in situ* production of bacteriocin with the additional benefit of increasing the physical, chemical as well as sensory qualities of such products [5]. Strains of both *Enterococcus faecium* and *Enterococcus faecalis* isolated from food and dairy products have proved their worth in this regard. To study the biochemical and antibacterial properties and to determine their effectiveness in food systems,

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it is necessary to obtain relatively large quantities of these peptides in pure and concentrated form.

Enterococci are also used as starter cultures in some cheeses and even as animal and human probiotics. In spite of this, many food hygienists are reluctant to accept the presence of enterococci in food because there is no consensus as to whether they can be generally recognized as safe micro-organisms, despite their long history in foods without causing health concerns.

The present investigation was focused on the characterization of enterocin from *Ent. faecium* FH 99, an isolate of human origin inhabiting its natural habitat. For successful exploitation of this strain, the strain was also physiologically investigated for its virulence potential.

Materials and methods

Bacterial cultures

The bacterial strains used in this study, their source and the media employed are listed in Table 1. Enterococci were isolated from human faeces. Samples were collected as faecal swabs and diluted samples were streaked on Modified Citrate Azide Agar [6]. The plates were incubated aerobically at 37°C for 48 h, and then several colonies were randomly picked and screened for bacteriocinogenic activity by the method of Uhlmann *et al.* [7] by using the indicator culture of *Pediococcus acidilactici* LB 42 (a sensitive strain used for detection of bacteriocin producers), kindly provided by Prof. Bibek Ray (Department of Animal Science, University of Wyoming, Laramie Wyoming, USA). After incubation at 37°C the plates were examined for zones of inhibition. Cultures were maintained in the MRS broth [8] at 4°C and before use they were propagated twice in their respective broth media and at their respective temperature of incubation.

Taxonomic identification

The isolate FH 99 was selected as bacteriocin producer because of its maximum bacteriocin titres against *Ped. acidilactici* LB 42. The strain was subjected to phenotypic and genotypic identification. Cell morphology and Gram-staining reaction were examined by light microscopy. Test for catalase activity was carried out. Phenotypic identification was based upon biochemical characteristics, including the ability of the strain to grow at 10 and 45°C, in the presence of 6.5% (w/v) NaCl, on bile-aesculin agar, and in the presence of 0.04% K-tellurite. Finally, identification was genotypically confirmed by species specific PCR. Total DNA from the strain FH 99 obtained using the method of

Pospiech and Neumann [9] was used as a template and the specific primers EM1A: ttgaggcagaccagattgacg and EM1B: tatgacagcgactccgattcc described by Cheng *et al.* [10] (custom synthesized by Bangalore Genei, Bangalore, India) were used for carrying out PCR amplification of target gene. Reaction mixture consisted of 1X PCR buffer, 200 μ M concentration of deoxynucleoside triphosphate mix, 0.25 μ M concentration of each primer (Bangalore Genei, Bangalore, India), 50 ng bacterial DNA, and 0.04 U μ l⁻¹ Taq DNA polymerase (Bangalore Genei, Bangalore, India). The PCR cycling conditions used in this study for *Ent. faecium* included initial denaturation at 95°C for 4 min and 35 cycles each of denaturation at 94°C for 45 sec followed by annealing at 51°C for 45 sec and extension at 72°C for 45 sec followed by final extension at 72°C for 7 min. The amplification product of the isolate FH 99 was got sequenced by the services provided by Bangalore Genei Pvt. Ltd., Bangalore, India to confirm results of species specific PCR.

Bacteriocin activity

The inhibitory spectrum of activity was obtained using the agar-well assay [11] against the strains listed in Table 1. Fifty microlitres of the cell free culture supernatant (CFCS) of an overnight culture of strain FH 99 grown in MRS broth, obtained after centrifugation at 10,000 rpm for 10 min at 4°C, was added to the wells punched in TGE agar plates [12] (1.5% agar). Before punching the wells, TGE agar plates were overlaid with TGE soft agar (0.75%) seeded with actively growing cells of the test organisms. Plates were kept undisturbed for 3–4 h for diffusion of CFCS through agar and then incubated. The sensitivity of the strain in question was evaluated by checking for clear zones around wells.

Detection of virulence determinants

Presence of the virulence characters *viz.*, Hemolysin production, DNase activity and gelatinase activity were determined as per the methods given by the workers [13].

Production of enterocin

Active culture of *Ent. faecium* FH 99 was inoculated at 1% in the MRS broth (initial pH-7.5). Incubation was carried out at 37°C for 24 h and CFCS were prepared. The titre of the bacteriocin activity (AU ml⁻¹) was expressed as the reciprocal of the highest dilution showing a definite zone of inhibition against the indicator *Ped. acidilactici* LB 42 by Spot-on-lawn assay [7].

Table 1 Bacterial species and strains used in the study

Bacterial species	Strains and Source	Culture conditions	Diameter of Zone of inhibition*
Gram-positive lactic acid bacteria			
<i>Pediococcus acidilactici</i>	LB-42, USA	37°C, MRS ^a	+++
<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	NCDC 86, Karnal	30°C, M17 ^b	+++
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	NCDC 278, Karnal	30°C, M17	+++
<i>Lactobacillus casei</i> subsp. <i>casei</i>	NCDC 017, Karnal	37°C, MRS	-
<i>Lactobacillus brevis</i>	NCDC 001, Karnal	37°C, MRS	+++
<i>Lactobacillus acidophilus</i>	NCDC 291, Karnal	37°C, MRS	+++
<i>Lactococcus cremoris</i>	NCDC 060, Karnal	37°C, MRS	+++
<i>Lactobacillus helveticus</i>	NCDC 005, Karnal	37°C, MRS	+++
<i>Lactobacillus fermentum</i>	NCDC 156, Karnal	37°C, MRS	+++
<i>Lactobacillus rhamnosus</i>	NCDC 018, Karnal	37°C, MRS	+++
<i>Lactobacillus plantarum</i>	NCDC 020, Karnal	37°C, MRS	++
<i>Lactobacillus paracasei</i>	NCDC 063, Karnal	37°C, MRS	+++
<i>Lactobacillus lactis</i> 11	Lab. isolate	37°C, MRS	+++
<i>Lactobacillus brevis</i> 21	Lab. isolate	37°C, MRS	-
<i>Lactobacillus brevis</i> 22	Lab. isolate	37°C, MRS	-
<i>Lactobacillus brevis</i> 23	Lab. isolate	37°C, MRS	-
<i>Lactobacillus brevis</i> 24	Lab. isolate	37°C, MRS	-
<i>Lactobacillus helveticus</i> 2	Lab. isolate	37°C, MRS	+++
<i>Streptococcus thermophilus</i>	NCDC 074, Karnal	37°C, M17	+++
<i>Streptococcus thermophilus</i>	NCDC 117, Karnal	37°C, M17	+++
<i>Streptococcus thermophilus</i>	NCDC 80, Karnal	37°C, M17	+++
<i>Enterococcus faecalis</i>	DSM 20478, Germany	37°C, MRS	+++
<i>Enterococcus faecalis</i>	NCDC 115, Karnal	37°C, MRS	+++
<i>Staphylococcus aureus</i>	NCDC 110, Karnal	37°C, BHI ^c	++
<i>Listeria monocytogenes</i>	Scott A, USA.	37°C, BHI	+++
<i>Micrococcus luteus</i>	NCDC 131, Karnal	37°C, M17	+++
Gram-positive non-lactic bacteria			
<i>Staphylococcus aureus</i> VR17	Lab. isolate	37°C, BHI	+
<i>Staphylococcus aureus</i> 29	Lab. isolate	37°C, BHI	++
<i>Streptococcus agalactiae</i> 19	Lab. isolate	37°C, BHI	+++
<i>Streptococcus agalactiae</i> 2	Lab. isolate	37°C, BHI	+++
<i>B. subtilis</i>	Lab. isolate	37°C, NB ^d	-
Gram-negative pathogens			
<i>Klebsiella pneumoniae</i>	NCDC 138, Karnal	37°C, BHI	-
<i>Salmonella typhi</i>	NCDC 113, Karnal	37°C, BHI	-
<i>Shigella dysenteriae</i>	NCDC 107, Karnal	37°C, BHI	-
<i>E. coli</i>	NCDC 134, Karnal	37°C, BHI	-
<i>E. coli</i> V517	Prof. Bibek Ray, USA	37°C, BHI	-
<i>Pseudomonas aeruginosa</i>	NCDC 104, Karnal	37°C, BHI	-

*Agar Well assay for *E. faecium* FH 99; +++ = >15 mm; ++ = 10–15 mm; + = < 10 mm.

^aMRS = de Man, Rogosa, Sharpe.

^bM17 = Terzaghi and Sandine [41].

^cBHI = Brain Heart Infusion (HiMedia Laboratories, Mumbai, India).

^dNB = Nutrient Broth.

Sensitivity to enzymes, heat treatment and buffers of different pH values

CFCS from an early-stationary-phase culture of strain FH 99 was tested for stability in the presence of several physicochemical agents and sensitivity to enzymes, using *Ped. acidilactici* LB 42 as indicator strain.

Enzyme susceptibility: For enzyme susceptibility assays, active supernatants were incubated at 37°C for 2 h in the presence of 1 mg ml⁻¹ of protease I and XIII, proteinase K, pepsin, chymotrypsin, trypsin, pronase-E, papain, ficin, rennin, catalase, amylase or lipase, dissolved in buffers as recommended by the suppliers (Sigma-Aldrich, St. Louis, Missouri, USA). Untreated bacteriocin and enzyme solutions were used as controls.

Stability to heat: To evaluate thermostability, aliquots of CFCS were heated to 60°C/30 min, 80°C/10 min and 100°C/10 and 30 min and 121°C/15 min, immediately cooled in ice and tested for antimicrobial activity.

Stability at different pH: CFCS was dissolved in 1:1 ratio in sterile buffers (pH 2–10) prepared at double concentration (200 mM) and was incubated at 37°C for 2 h. pH was neutralized to pH 7.0 to test for residual activity. Buffer solutions (100 mM) without supernatants were used as control.

Heat stability at different pH: The CFCS adjusted to different pH values 3, 6 and 9, were subjected to four different heat treatments viz. 60°C/10 min, 60°C/30 min, 100°C/15 and 121°C/15 min, cooled on ice immediately and the residual activity of both the preparations was determined after 8 h of storage at room temperature.

In all the cases the residual activity of treated and untreated samples was determined by the spot-on-lawn assay against the indicator strain *Ped. acidilactici* LB42.

Enterocin purification

E. faecium FH 99 was grown in at 37°C for 18 h (OD₆₂₀ 1.4) in 1 l of the buffered MRS broth (initial pH 7.5) till late-exponential phase. CFCS was prepared and the bacteriocin activity (AU ml⁻¹) and protein content [14] of the supernatant were determined to calculate the specific activity. Crude enterocin FH 99 was precipitated from broth media by 60% ammonium sulphate precipitation and the precipitates were dissolved in sterilized Milli Q water. The specific activity of the ammonium sulphate preparation was calculated. The specific activity of the enterocin was compared for three different purification techniques as below:

Gel filtration: The bacteriocin preparation obtained after ammonium sulphate precipitation was purified by gel filtration chromatography. Suprose-12 column (30 × 10 mm) obtained from Pharmacia GE Health Sciences,

Uppsala Sweden was equilibrated for 3–4 h with 50 mM acetate buffer containing 100 mM KCl (pH 5.0). With the help of a syringe, 0.5 ml of the 60% ammonium sulphate preparation was applied on to the column and the elution of bacteriocin was carried out at a flow rate of 0.33 ml min⁻¹. Fractions were collected after draining 80% of the void volume. Fractions pertaining to different peaks were collected in different tubes and were analyzed for bacteriocin activity. The protein content of the fractions was monitored by determining OD at 280 nm.

Cation-exchange chromatography: Partially purified bacteriocin preparation after ammonium sulphate precipitation was subjected to cation exchange chromatography on SP-Suprose Fast Flow Column (10 cm × 26 mm; Pharmacia GE Health Sciences, Uppsala, Sweden). Solvent A used was 20 mM acetate buffer (pH 5.0), and solvent B was 20 mM acetate buffer with 1M KCl (pH 5.0), both filtered through 0.45 µm membrane filter and degassed before use. Column was thoroughly washed before use and equilibrated with Solvent A. The bacteriocin was eluted from the column with a linear gradient (1–100%) of solvent B at a flow rate of 1.5 ml/min. Different peaks were collected in tubes and checked for bacteriocin activity.

RP-HPLC: The reverse-phase HPLC analysis of partially purified bacteriocin from ammonium sulphate precipitation also was done on C18 analytical column (25 × 4.6 mm, Shimadzu, Japan). Solvent A used was 0.1% trifluoroacetic acid (TFA; Sisco Research Laboratories, Pvt. Ltd., Mumbai, India) in HPLC grade water. Solvent B was 0.1 % TFA in 100% acetonitrile (Sisco Research Laboratories, Pvt. Ltd., Mumbai, India). Both the solvents were filtered through 0.45 µm membrane filter and degassed before use. TFA was added to the sample at a concentration of 0.1 %. After centrifugation at 14,000 rpm for 10 min, 20 µl of the sample was applied to the column. Sample was centrifuged to remove out any precipitates. The bacteriocin was eluted from the column with a gradient of acetonitrile in aqueous 0.1% (v/v) TFA as: 0–40% for 20 min, 40–80% for 20–50 min and 80–100% for 50–70 min at a flow rate of 0.5 ml/min. Fractions were checked for bacteriocin activity.

Molecular weight approximation

Approximation of the molecular weight of the partially purified enterocin FH 99 sample was determined by SDS-PAGE as described by Laemmli [15] on 18% slab gels. After electrophoresis, one half of the gel was stripped off, fixed and stained with Coomassie Blue R 250. The other half containing same sample was fixed for 2 h at room temperature in 15% (v/v) methanol and 10% (v/v) glacial acetic acid, and then exhaustively washed in deionized water for

12 h. Finally the gel was placed on a sterile Petri dish with 15 ml of TGE agar and overlaid with 7 ml TGE soft agar previously seeded with an active culture of *Ped. acidilactici* LB 42 (approx. 10^7 CFU ml⁻¹). The plate was incubated for 18 h at 37°C and examined for zone of inhibition.

Plasmid isolation from *Ent. faecium* FH 99

The protocol of Sullivan and Klaenhammer [16] was followed for extraction of plasmids from the culture prepared in MRS broth. Plasmid profiles were obtained by electrophoresis in a horizontal gel apparatus (Mini submarine, Hoeffer, USA) on a 0.7% agarose gel at 40 V for 4–5 h. The gel was illuminated with UV light and a digital image was created using BioRad gel Doc (Bio-Rad, Hercules, CA, USA).

Plasmid curing

An overnight grown culture of *Ent. faecium* FH 99 in MRS broth was adjusted to approx. 10^5 cells ml⁻¹ using fresh MRS medium and incubated for 72 h at 45°C with/without curing agents viz., ethidium bromide (5–50 µg ml⁻¹), acridine orange (5–50 µg ml⁻¹), acriflavin (5–50 µg ml⁻¹) or nalidixic acid (5–50 µg ml⁻¹) (HiMedia Laboratories, Ltd., Mumbai, India). Cells treated with the highest concentration of curing agents displaying visible growth were diluted in sterile saline and plated onto MRS agar. After overnight incubation at 37°C, colonies were randomly picked and screened for Bac⁻ variants using *Ped. acidilactici* LB 42 as indicator strain. After an additional overnight incubation at 37°C, inhibitory capacity as well as plasmid content in bacteria was tested. Parent (Bac⁺) culture was used as a control.

Results

Identification of the enterocin FH 99 producer strain

Faecal samples of human were found to be potential source for the isolation of bacteriocinogenic enterococci. Among the various bacteriocinogenic enterococcal isolates *Ent. faecium* FH 99 was found to show maximum bacteriocin titres against indicator *Ped. acidilactici* LB 42.

Isolate FH 99 was identified on the basis of its morphological and biochemical characteristics. This strain was found to be Gram-positive coccus, catalase and oxidase negative isolate with ability to grow at 10 and 45°C, at pH 9.6 and growth in the presence of bile-esculin and 6.5% NaCl. The isolate was, therefore, identified as *Enterococ-*

cus. Finally, according to the sugar fermentation pattern the isolate was assigned to the species *Ent. faecium/Ent. casseliflavus*. Genotypic characterization by PCR amplification confirmed it to be *Ent. faecium* as a specific amplification product of 658 bp was obtained (Fig. 1). The clean readable sequence of 578 bp has been submitted to Genbank (Accession No. EF694229). Sequence similarity search for this sequence (Blast, NCBI) showed 93% homology with the nucleotide sequence of available gene of *Ent. faecium* Accession No. L78127[10] and 94% homology with partial nucleotide sequence of ATP dependent DNA helicase RecG gene of *Ent. faecium* DO Accession No. NZ_AAAK0300012 (Fig. 2). Whereas at the amino acid level 100% homology with partial sequence of ATP dependent DNA helicase RecG protein of *Ent. faecium* DO Accession No. ZP_00605048 (Fig. 3) was observed. The amino acid sequence obtained from nucleotide Accession No. L78127 revealed 98% homology with our protein. The targeted gene for PCR amplification was, therefore, identified as a part of gene coding partial sequence of ATP dependent DNA helicase RecG protein of *Ent. faecium*.

Fig. 1 Electrophoretic profile of bacteriocinogenic isolates of *Ent. faecium* on PCR amplification with primers targeted to ATP dependent DNA helicase *recG* gene. Lane M - Marker (100bp, BangaloreGenei, Bangalore, India); Lane 1 Reference culture *Ent. faecium* BFE 900; Lane 2 - *Ent. faecium* FH 99.

Fig. 2 Neighbour Joining Tree (NJ Tree) showing relationship among nucleotide sequences. Relationship among nucleotide sequences of isolate FH 99 and the nucleotide sequences of the gene of *Ent. faecium* with Accession No. L78127 (Cheng *et al.*, 1997) and nucleotide sequence of ATP dependent DNA helicase RecG gene in *Ent. faecium* DO (Accession No. NZ_AAAK03000121).

Detection of virulence determinants

The strain under study did not show any haemolytic, DNase or gelatinase activity.

Inhibitory spectrum of *Ent. faecium* FH 99

The bacteriocin of the strain *Ent. faecium* FH 99 displayed a wide inhibitory spectrum against many Gram positive bacteria including *Listeria monocytogenes* and some strains of *Staph. aureus* (Table 1). Bacteriocin of *Ent. faecium* FH 99 strain did not show inhibitory activity against Gram-negative organisms viz., *E. coli*, *Shigella* sp., *Salmonella enteritis*.

Production of bacteriocin

The highest activity of bacteriocin (1.8×10^5 AU ml⁻¹) was detected during the late log-phase (after 18 h) in MRS medium at 37°C and thereafter, a little decrease in bacteriocin

titres was observed (Fig. 4). Production of enterocin was assayed using *Ped. acidilactici* LB 42 as an indicator strain.

Stability of inhibitory substance

The antimicrobial substance produced by *Ent. faecium* FH 99 appeared to be heat-stable, pH stable and insensitive to enzymes catalase, amylase, lipase or even rennin

Table 2 Effect of different enzymes, pH and temperature on inhibitory activity of enterocin produced by *Ent. faecium* FH 99

Treatment	Residual Activity (AU ml ⁻¹)
Enzyme treatment	
Control	90,000
Catalase	90,000
Proteinase K	0
Pepsin	0
Papain	0
Trypsin	0
Pronase-E	0
Ficin	0
Rennin	90,000
Lipase	90,000
-Amylase	90,000
Protease XIII	0
Chymotrypsin	0
Protease I	0
Heat Treatment	
Control	90,000
63°C/30 min	90,000
85°C/10 min	90,000
85°C/15 min	90,000
90°C/10 min	90,000
90°C/15 min	90,000
100°C/5 min	90,000
100°C/10 min	90,000
100°C/30 min	90,000
100°C/60 min	80,000
121°C/15 min	80,000
pH stability	
Control	90,000
pH 1	90,000
pH 2	90,000
pH 3	90,000
pH 4	90,000
pH 5	90,000
pH 6	90,000
pH 7	90,000

Fig. 3 Neighbour Joining Tree (NJ Tree) showing relationship among amino acid sequences. Relationship among amino acid sequences of isolate FH 99 (HG.99B.EMIAF) and the amino acid sequences of ATP dependent DNA helicase RecG protein (Rec) in *Ent. faecium* DO (Accession No. ZP_00605048) and amino acid sequence of protein coded by gene (L78127 Cheng) with accession No. L78127 (Cheng et al. 1997).

Fig. 4 Changes in growth pH and bacteriocin production by *E. faecium* FH 99 under optimized conditions for maximum bacteriocin production. Growth (—) was followed by optical density (OD₆₂₀), production of Ent FH 99 (—) determined by the spot-on-lawn and expressed in arbitrary units (AU ml⁻¹). Changes in pH during growth (---).

Table 2 Continued

pH 8	90,000
pH 9	90,000
pH 10	65,000
Heat stability at different pH	
Control	90,000
60°C/10 min	
pH 3	90,000
pH 6	90,000
pH 9	90,000
60°C/30 min	
pH 3	90,000
pH 6	90,000
pH 9	90,000
100°C/15 min	
pH 3	90,000
pH 6	90,000
pH 9	70,000
121°C/15 min	
pH 3	80,000
pH 6	60,000
pH 9	50,000

(Table 2), however, it was completely inactivated by all proteolytic enzyme treatments *viz.*, proteinase K, pepsin, papain, trypsin, pronase-E, ficin, protease XIII, chymotrypsin and protease I.

Enterocin purification

The bacteriocin was eluted from the column in the first few fractions immediately after the void volume in Gel filtration chromatography. Bacteriocin activity of the fractions is shown in Fig. 5. The elution profile of enterocin FH 99 with linear gradient of 1M KCl in cation exchange chromatography is also presented in Fig. 6. Bacteriocin was eluted at higher ionic strength of the salt (>60% concentration of eluent) and showed a very small peak corresponding to protein content $78 \mu\text{g ml}^{-1}$ for FH 99. During purification by RP-HPLC, enterocin FH 99 retained in a reverse-phase C18 column, was eluted at a high solvent concentration (Fig. 7). This chromatography revealed one major peak after 27 min and the activity was observed in fractions collected after 27.6 min to 37 min. However, the recovery of the protein in terms of activity was quite less in all the three chromatographic techniques used (Table 3).

In gel filtration and RP-HPLC, a major peak possessing antibacterial activity was recovered whereas in cation exchange chromatography the antibacterial activity was possessed by minor peak.

Fig. 5 Purification of enterocin FH 99 by gel filtration chromatography. Bold line shows the protein content in terms of OD at 280 nm and dotted line shows the peak pertaining to bacteriocin activity.

Fig. 6 Purification of enterocin FH 99 by cation-exchange chromatography. Bold line shows the protein content in terms of OD at 280 nm and dotted line shows the peak pertaining to bacteriocin activity.

Fig. 7 Purification of enterocin FH 99 by RP-HPLC. Bold line shows the protein content in terms of OD at 280 nm and dotted line shows the peak pertaining to bacteriocin activity.

Table 3 Purification of enterocin FH 99 from culture supernatant of *Ent. faecium* FH 99

Sample	Total Activity Units (AU X 10 ³)	Total Proteins (mg)	Specific Activity ^a (X 10 ³ AU mg ⁻¹)	Purification fold ^b	Recovery ^c
Culture Supernatant	1,80,000	1270	141.73	1	100%
60% Ammonium sulphate precipitation	1,20,000	85	1411.76	9.96	66.67%
Gel filtration chromatography	150	2.73	54.94	0.38	0.12%
Cation-exchange chromatography	100	0.42	238.09	1.68	0.08%
RP-HPLC	40	1.54	25.97	1.83	0.03%

^a total activity units divided by total proteins.^b purification fold relative to specific activity of culture supernatant.^c Activity Units ratios. **Fig. 8** Determination of molecular weight of partially purified enterocin FH 99 by SDS-PAGE. (a). Gel stained with brilliant blue G; M-Marker (size in kb); FH 99- band of enterocin FH 99 (b). Unstained gel depicting the *in-situ* bacteriocin activity.

Determination of molecular weight

The gel stained with the dye had shown one protein band with molecular weight <6.5 kDa (Fig. 8). Superimposing of stained gel over the unstained gel used to detect the bacteriocin activity revealed that the <6.5 kDa protein band detected in the stained gel corresponded to enterocin FH 99.

Plasmid profile of bacteriocinogenic *Ent. faecium* FH 99

It may be seen from the Fig. 9 that *Ent. faecium* FH 99 strain harbour two plasmids (Lane 1) of size >33.5 kb (greater than the chromosomal DNA).

Curing of plasmids

Incubation of the parent strains at 45°C in the presence of different concentrations of intercalating agents such as acridine orange (50–200 µg ml⁻¹), acriflavine (5–50 µg ml⁻¹), ethidium bromide (5–20 µg ml⁻¹) and nalidixic acid (5–20

Fig. 9 Agarose gel electrophoresis of plasmid DNA. (a). Six plasmids were detected in *Ent. faecium* FH 99 (lane 1). Two plasmids of size > 33.5 kb were observed; lane 2- Supermix DNA ladder (Bangalore Genei, Pvt Ltd., Bangalore) (b). Plasmid profile of Bac. variants; no plasmid was detected in Bac- variants (lane 2–4).

µg ml⁻¹) failed to yield bac⁻ variants of *Ent. faecium* FH 99. Only three variants (FH 99N1, FH 99N2 and FH 99N3) could be obtained by treatment of cells of *Ent. faecium* FH 99 with higher concentration of ethidium bromide (50 µg ml⁻¹). This indicates the highly stable nature of the plasmid bearing Bac⁺ gene. The inability of the cured variants to produce bacteriocin was confirmed by growing them in MRS broth at 37°C for 18 h and assessing the bacteriocin activity of the heat killed culture broths against *Ped. acidilactici* LB 42 by agar-spot-assay.

Plasmid profile of cured Bac⁻ variants

Plasmid profile of the parent FH 99 strain and all the 3 Bac⁻ variants is shown in Fig. 9(b). All Bac⁻ variants of

Ent. faecium FH 99 were devoid of plasmids (Lane 2 to 4), whereas plasmids were present in the parent strain *Ent. faecium* FH 99 (Lane 1). It might be, therefore suggested that production of bacteriocin is encoded by genes present on any of these two plasmids. Cured Bac⁻ variants *Ent. faecium* FH 99N1, FH 99N2 AND FH 99N3 were sensitive against bacteriocin of *Ent. faecium* FH99 suggesting that their immunity genes have also been lost. This loss in both the bacteriocin production and immunity trait indicate that these characters may be plasmid encoded.

Discussion

Enterococci are present in a variety of foods and in some of them they play important role in fermentation and ripening. Some strains offer potential for use as starter, adjunct or protective cultures especially in dairy industry [17] and meat industry [18]. Furthermore, some strains of *Ent. faecium* and *Ent. faecalis* are known probiotics and have been used with great success in commercial probiotic preparations [19, 20]. In addition, many different bacteriocins have been described in enterococci, which are of great interest for food preservation against food borne pathogenic and spoilage bacteria. In recent years, several *Ent. faecium* and *Ent. faecalis* strains from dairy products and/or silage displaying antibacterial activity have been isolated [20–22]. Isolation of bacteriocinogenic enterococci from faeces of human and animal have also been well reported [23–25]. Our findings are in agreement with these studies showing that bacteriocin producing enterococci can be found associated with gastrointestinal tract of human.

Nowadays, ability of enterococci to inhibit *Listeria* spp. is well known, which may be explained and accentuated by the close phylogenetic relationship of enterococci and *Listeriae* [3, 20]. *Ent. faecium* FH 99 has been found to possess inhibitory activity against *Listera monocytogenes* Scott A besides activity against *Staphylococcus aureus* and other spoilage lactic acid bacteria. Like the findings of other authors [26], our findings also suggested that bacteriocins of LAB are primarily active against Gram-positive organisms. A few reports, however, do indicate enterocins to be active against Gram-negative bacteria such as *E. coli* [27, 28], *Shigella sonnei*, *Shigella flexneri* [29] and *Vibrio cholera* [30]. But in the present investigation, bacteriocin of *E. faecium* FH 99 was not found to be effective against Gram-negative organisms.

An ambiguity in the exact identity of the isolate FH 99 as *Ent. faecium*/*Ent. casseliflavus* was confronted through biochemical identification. But PCR amplification of the specific gene with the primer pairs (EM1A/ EM1B) devised by Cheng *et al.* [10] helped to confirm its identity

as *Ent. faecium*. The product obtained on PCR amplification by using this primer pair codes for the protein ATP dependent helicase RecG gene. The target gene had not been revealed for these primer pairs and the protein it codes for. Hardwood *et al.* [31] have also used these primer pairs for the identification of *Ent. faecium* but have not sequenced the product. In our studies, through blast search, we have found that the PCR product obtained by using primer pairs EM1A/ EM1B is a part of the gene that codes for ATP dependent helicase RecG protein.

Bacteriocin production by the isolate *Ent. faecium* FH 99 was observed at maximum levels at high cell densities. Various other workers have also reported the maximum bacteriocin production at late log phase along with decreased bacteriocin activity after 48 h of incubation. The decrease in activity has been suggested due to culture proteases or readsorption of bacteriocin to the producer cell surface at low pH [29, 32].

The sensitivity of bacteriocins of LAB to various proteolytic enzymes and insensitivity to non-proteolytic enzymes has been reported widely [21, 29]. Bacteriocin of FH 99 was found to retain its activity after treatment with rennin. This suggests that this bacteriocin producer/ bacteriocin can be a suitable candidate for biopreservation of cheese. The retention of the bacteriocin activity after different heat treatments and activity over a wide range of pH shows that the bacteriocin FH 99 can be employed in acidic as well as alkaline foods. The extreme heat stability is believed to be because of their simple structure and more solubilities at low pH values [33].

Bacteriocins of enterocins in general, are reported to be cationic hydrophobic peptides except enterocin MR99, an anionic peptide produced by *Ent. faecalis* MR99[29] and have been purified by cation-exchange chromatography to a greater extent [21, 34, 35]. Several workers have reported >50% recovery of antibacterial activity by gel filtration and cation-exchange chromatography of the crude bacteriocin [29, 35]. In our study we got <1% recovery in terms total activity and specific activity values. Hechard *et al.*[36] had also reported only 0.7% recovery of the antibacterial peptide-Mesentericin Y105 produced by *Leuconostoc mesenteroides* Y105 after purification by affinity chromatography, ultrafiltration and reverse-phase high-pressure liquid chromatography. Ganzle *et al.* [37] had demonstrated that ecological factors, such as protein content, pH, salinity, and/or salt composition of the medium modulate the activity of bacteriocins.

The enterocin in this study exhibited low molecular weights of approximately 5 kDa. It could, therefore, be tentatively classified as class II bacteriocin [5]. The low molecular weight of enterocin in this study is in accordance

with molecular weight of enterocins reported by various workers [21, 29], but they differed from the higher molecular weight enterocins described by others [3, 38].

Presence of large size of plasmids in the enterococci like other lactic acid bacteria seem to be common characteristic as reported by several other workers. Large size plasmids of size 49 kb and >70 kb respectively, along with other low molecular weight plasmids have been reported in enterococci by various workers [21, 39].

In an attempt to determine, whether the bacteriocin production trait was chromosomally encoded or plasmid encoded, the Bac⁺ *Ent. faecium* FH 99 was propagated at 45°C and also in the presence of various curing agents at 45°C. Plasmid-free derivatives could be obtained only after prolonged curing at 45°C. High temperature treatment alone failed to generate any Bac⁺ variants may be because of their high molecular weight. Other workers also reported the same results [39, 40]. Our findings are very much similar to the findings of the workers who have reported that bacteriocin production in *Ent. faecium* strains is generally plasmid encoded [21, 29, 38, 39]. However, chromosomally encoded bacteriocins are not uncommon among lactic acid bacteria [11, 40].

Conclusions

Enterococci are ubiquitous in nature and are present in gut of humans. Their capacity to produce bacteriocins active against spoilage or pathogenic bacteria, particularly against *Listeria*, might be a powerful tool for the protection of certain dairy products and meat products against such pathogens. A careful evaluation of the commercially exploitable strains may be very helpful for the food industry for their safe use. Present investigation has afforded a bacteriocinogenic strain of *Ent. faecium* FH 99 that is quite unique for its very high bacteriocin activity and inhibition towards *Listeria* and other food spoilage and pathogenic bacteria. Bacteriocin of this strain is quite heat stable and active over a wide range of pH indicating that it can be exploited in the biopreservation of acidic as well as non-acidic foods. Resistance of this bacteriocin towards the action of rennin makes it a suitable candidate for its use in cheese manufacturing process. A careful evaluation of this strain for its technological performance may help in its successful exploitation as starter, adjunct or protective culture.

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