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ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF ENTEROCIN ESF100 PRODUCED BY *ENTEROCOCCUS FAECALIS* ESF100 ISOLATED FROM A PATIENT SUFFERING FROM URINARY TRACT INFECTION

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Abstract

Enterococcus faecalis ESF100 has been found to produce antibacterial substance known as enterocin ESF100. The inhibitory activity could not be related to organic acids, bacteriophages and hydrogen peroxide. It has a broad-spectrum activity against many Gram+ve bacteria but less active against Gram-ve bacteria. It could be stored at 4^{o}C for 2 months without loss of activity and remained stable at 60, 80, 100^{o}C and autoclaving for 60 min., 40 min., 30 min., and 15 min., respectively. It is stable from pH 5-8.5. Enterocin ESF100 is resistant to treatment with chloroform, ficin, lipase, lysozyme and pepsin A, but partially sensitive to trypsin and fully sensitive to pronase E and chymotrypsin. Its production starts in early logarithmic phase and reaches to maximum between 2½-4 h and then remains constant throughout incubation. It has bactericidal effect and causes rapid cessation of the growth of the sensitive strains. Its production is regulated by the genetic factors located on the extra-chromosomal element (plasmid) pESF100 having molecular weight > $1.4x10^{7}$Da.

Introduction

Enterocin production by group D streptococci has been well documented for most of the enterococcus bacteria. Several enterocins produced by enterococci have so far been characterized. Some of them have a narrow inhibitory spectrum e.g., enterocin 226NWC from *E. faecalis* 226 (Villani *et al.*, 1993) and enterocin 4 from *E. faecalis* INIA4 (Joosten *et al.*, 1996). The ability of enterococci to produce broad-spectrum activity against both Gram+ve and Gram-ve bacteria is also well known. Enterocin CCM4231 a well characterized bacteriocin from *E. faecium* (Laukova *et al.*, 1993); enterocin 012 from *E. gallinarum* (Jennes *et al.*, 2000) and enterocin V24 from *E. faecalis* V24 (Laukova *et al.*, 2003). Three *E. faecium* strains (JBLI061, JBLI083 and JBLI351) have been shown to exhibited strong anti-listerial activity against *L. monocytogenes.* The antagonistic factors produced by these three strains were found sensitive to chloroform and several proteolytic enzymes (Arihara *et al.*, 1993). Similarly, enterocin A from *E. faecium* EFM01 also showed anti-listerial activity and was found stable between pH 4-9 (Ennahar & Deschamps 2000). Enterococci possessed extra-chromosomal genetic elements for drug resistance, hemolysin and bacteriocin production. These elements (plasmids) were able to move from one strain to another via conjugation (Yasuyoshi & Clewell 1992). Purification and N-terminal amino acid sequencing of enterocin CRL35 produced by *E. faecium* CR35 was studied by (Farias *et al.*, 1996). Enterocin CR35 showed homology of amino terminal sequence with other bacteriocin of LAB bacteria. Two antimicrobial peptides namely enterocin 1071A and enterocin 1071B produced by *E. faecalis* BFE 1071 were found to have a molecular mass of 4.285KDa and 3.899KDa respectively. Genes of both the enterocins were located on 50Kbp plasmid (Balla *et al.*,

2000). Bacteriocin like activity by enterococci has more recently been reported and these enterocins displayed strong inhibitory action against *Listeria monocytogenes* (Herranz *et al*., 2001). Enterocin production by *E. faecium* RZC5 was high at pH 6.5 (Foulquie *et al.*, 2003). Genes of enterocin EJ97 is located on conjugative plasmid, which is a cationic bacteriocin of (5,327.7Da), its complete amino acid sequence was also elucidated (Sanchez-Hidalgo *et al.,* 2003). The present report describes the characterization of enterocin ESF100, a bacteriocin produced by *E. faecalis* ESF100, which has a broad-spectrum antagonistic activity.

Materials and Methods

Bacterial strains and growth conditions: *Enterococcus faecalis* ESF100 isolated from urine of a patient suffering from urinary tract infection (UTI) was used as producer of enterocin ESF100. Culture was grown on blood agar base (BAB, Oxoid) supplemented with 5% sheep blood and was sub-cultured once a week. Enterocin activity was checked on brain heart infusion agar (BHI, Oxoid) and tryptic soy agar (TSA, Oxoid). The sensitive/indicator cultures used in this study were Gram+ve, Gram-ve and yeast microorganisms. Producer and sensitive cultures were maintained in vials by growing them in 3mL of BHI broth and after 24h incubation overlaid with 2mL of 40% glycerol.

Enterocin activity: Two methods according to (Rasool *et al*., 1996) were used for the detection of enterocin activity. (1) Stab-overlay method. (2) Agar-well diffusion method.

Detection of lytic bacteriophages: Reverse-side technique was applied, which prevents the contact of bacteriophages with the producer and the indicator strains. The procedure culture was stabbed on BHI agar, and next day the indicator strain was poured on the face opposite to the one where the producing strain was grown, so that contact between indicator and producing strain was not possible (Parrot *et al*., 1990).

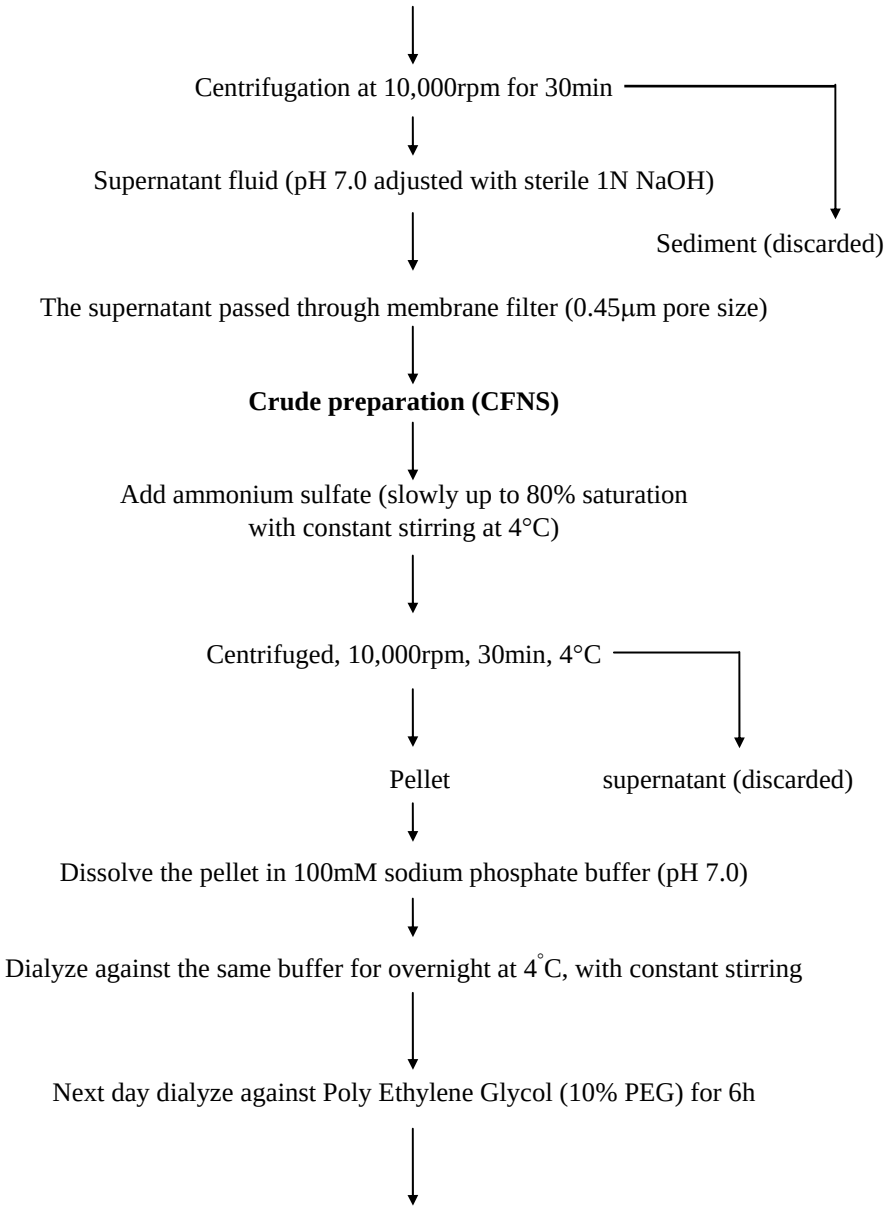
Inhibitory activity due to hydrogen peroxide or lactic acid production: To rule out the inhibitory activity of the enterocin ESF100 by hydrogen peroxide or lactic acid production, the method of (Muriana & Klaenhammer 1991) was followed.

Growth curve with simultaneous measurement of enterocin ESF100 production: Synthesis of enterocin ESF100 was monitored during the growth cycle by growing the producer culture *E. faecalis* ESF100 in BHI broth under shaking condition (150 rpm). After every half an hour optical density at 600nm was recorded, 0.1mL samples were plated on BHI agar medium to score the colony forming unit (cfu/mL) and 1.0mL samples were centrifuged (10,000 rpm, for 30 min) and supernatants were assayed for bacteriocin activity as described by (Iqbal *et al*., 1999).

Physico-chemical characterization

Thermostability: To check the thermal stability, CFNS was exposed to 60°C (60 min), 80°C (40 min), 100°C (30 min), 121°C (15 min) and 4°C (2 months) and activity was checked. (Iqbal *et al*., 2001).

18h old BHI broth culture of *E. faecalis* ESF100



Scheme 1. Schematic presentation of partial purification of enterocin ESF100 from *Enterococcus faecalis* ESF100.

pH stability: Enterocin preparations were adjusted to different pH levels between 2-12

with 10mM NaOH (Merck) or 10mM HCl (Merck). Samples were maintained for 2 h at 37°C. All the samples were then adjusted to pH 7.0 with sterile 4.0mM phosphate buffer and assayed for activity (Bhunia *et al*., 1991).

Effect of enzymes: Enzymes used in this study include: catalase, trypsin, chymotrypsin, pronase E, ficin, pepsin A, lysozyme and lipase. All enzymes were used at a final concentration of 1mg/mL (Muriana & Klaenhammer 1991). The enterocin preparations containing either of the enzymes were placed into wells and incubated at 37°C for overnight. Next day observed for presence or absence of zone of inhibition against *S aureus* AB211. Positive control (only bacteriocin) and negative control (only enzyme) was also run.

Effect of chloroform: To observe the effect of chloroform on the enterocin activity 24 h old stabbed culture was exposed to chloroform vapours for various timings (30 min) overlaid with appropriate sensitive cells and then next day zone size was measured (Rasool *et al*., 1996).

Lacuna assay*:* Lacuna assay was performed to determine the frequency of enterocin producing *E. faecalis* out of a whole population. For this reason method of (Rasool *et al*., 1996) was followed.

Mode of action: In order to ascertain the mode of action of enterocin ESF100 the viable count of logarithmic (growing) and stationary phase (non-growing) indicator cells was measured as per (Bhunia *et al*., 1988).

Curing & conjugation of enterocin plasmid: For curing, procedure of (Hardy 1993) was followed using SDS (2%) as curing agent. Plasmid mediated conjugation was done using *E. faecalis* ESF100 as donor and *S. aureus* AB222 and *E. coli* BU40 as recipient cultures.

Partial purification of enterocin ESF100: was done as per Iqbal *et al.,* 1999 (Scheme1).

Result and Discussion

Inhibitory spectrum of enterocin ESF100: *E. faecalis* ESF100 was screened for bacteriocinogenic potential by two different methods i.e. stab-overlay test and agar-well diffusion assay. The bacteriocinogenic activity of enterocin ESF100 against closely related and heterogeneous strains is given in (Table 1). It was found to be active against intrageneric and intergeneric strains. Interestingly, activity against intrageneric strains was observed only when tested by stab-overlay method, while no activity was observed in agar-well diffusion method. Most of the tested Gram-ve bacteria (*Agrobacterium tumefaciens*, *Enterobacter aerogenes*, *Escherichia coli*, *Proteus mirablis*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Kkebsiella pneumoniae* and *Shigella dysenteriae*) were not inhibited, while *Neisseria meningitidis* and *Xanthomonas maltophilia* were found sensitive. The broad host range antibacterial activity of *E. faecium* CCM4231 was earlier

Table 1. Spectrum of inhibition shown by *E. faecalis* ESF100 against Gram+ve and Gram-ve bacteria and yeast cells.

Sensitive/indicator organisms	Enterocin ESF100 activity	
	A	B
Gram+ve bacteria		
Bacillus subtilis	0/5	0
Bacillus cereus	0/1	0
Clostridium perfringens	0/1	0
Corynebacterium diphtheriae	1/1	20
Lactobacillus acidophilus	4/6	13
Micrococcus lysodiecticus	8/10	20
Listeria monocytogenes	5/6	14
Staphylococcus aureus	40/50	25
Staphylococcus epidermidis	4/12	10
Staphylococcus saprophyticus	1/1	13
Streptococcus agalactiae	3/5	15
Streptococcus equi	10/15	13
Enterococcus faecalis	6/10	14
Enterococcus faecium	6/10	14
Streptococcus mutans	30/30	16
Streptococcus pneumoniae	8/26	15
Streptococcus pyogenes	10/30	16
Streptococcus sanguis	4/4	15
Gram-ve bacteria		
Agarobacterium tumefaciens	0/1	0
Enterobacter aerogenes	0/1	0
Escherichia coli AB712	0/1	0
Escherichia coli BU40	0/1	0
Escherichia coli 5014	0/1	0
Escherichia coli WT	0/15	0
Klebsiella pneumoniae	0/12	10
Neisseria meningitidis	2/4	18
Proteus mirablis	0/1	0
Proteus vulgaris	0/1	0
Pseudomonas aeruginosa	0/17	0
Pseudomonas aeruginosa PAO286	0/1	0
Pseudomonas syringae	0/1	0
Salmonella typhi	0/5	0
Salmonella typhi para A	0/5	0
Salmonella typhi para B	0/5	0
Shigella dysenteriae	0/1	0
Shigella flexneri	0/1	0
Shigella sonnei	0/1	0
Xanthomonas maltophila	1/1	10
Yeast		
Candida albicans	0/8	0
Saccharomyces cerevisiae	0/2	0

A, Strains sensitive/Strains tested; B, Average zone size (mm); 0, No zone of inhibition

reported by (Laukova *et al.,* 1993; Jennes *et al.,* 2000) whereas, Ennahar & Deschamps 2000) had shown the narrow spectrum activity of the bacteriocins of enterococci. The activity unit (AU) of enterocin ESF100 was also determined and was found to be different against different sensitive strains (Table 2).

Table 2. Diffusion zone method for the titration of enterocin ESF100 against different indicator cultures.

Dilutions	Two-fold serial dilution of enterocin ESF100			
	A	**B**	**C**	**D**
Undiluted	+	+	+	+
1:2	+	+	+	+
1:4	+	+	-	+
1:8	+	-	-	+
1:16	+	-	-	+
1:32	+	-	-	+
1:64	+	-	-	+
1:128	+	-	-	+
1:256	-	-	-	+
1:512	-	-	-	+
***AU/mL**	**1280**	**40**	**20**	**5120**

+, Zone of inhibition; -, No zone of inhibition.

A, *Staphylococcus aureus* AB201; B, *Neisseria meningitides*, C, *Xanthomonas maltophila;* D, *Staphylococcus aureus* AB211

$$*\text{AU/mL} = \frac{\text{Reciprocal of the highest dilution x 1000}}{\text{Volume of bacteriocin}}$$

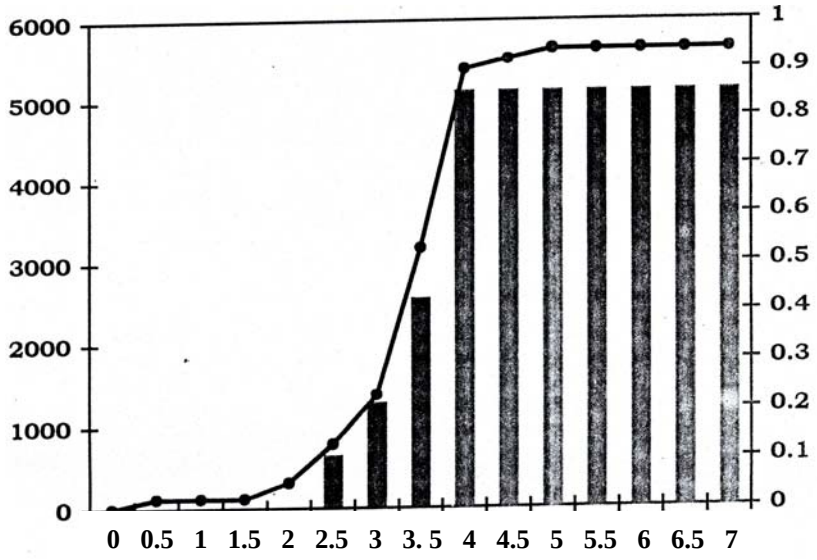


Fig. 1. Production of enterocin ESF100 during the growth cycle of *E. faecalis* ESF100. Samples were taken at different intervals and the Absorbance was measured at OD_{600}nm (shown by line) and enterocin production was determined in terms of AU/mL by agar-well diffusion assay (shown by bars).

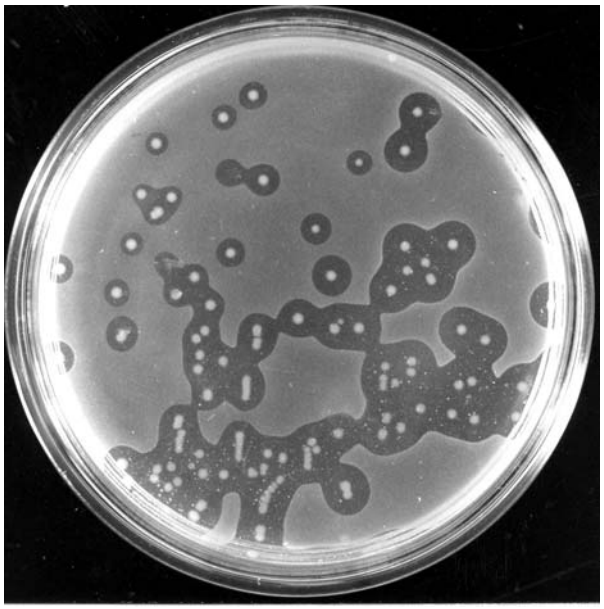


Fig. 2. Lacuna assay of Bac^+ strain of *E. faecalis* ESF100 against S. *mutans* VSMD. Assay differentiates between Bac^+ and Bac^- cells and between plaque forming bacteriophages and Bac^+ cells.

$$LF = \frac{LFC}{LFC+VBC}$$

where: LF, lacuna frequency; LFC, lacuna forming cells; VBC, viable bacterial cells

Activity other then lytic bacteriophages, hydrogen peroxide and lactic acid: The inhibition zones of the enterocin were still present even after seeding indicator culture in opposite direction, indicating that lytic bacteriophages were not responsible for these zones. The activity was retained when mixed with catalase (4000Units/mL) thereby, excluding the possibility of hydrogen peroxide and definite activity was obtained in stabbed culture as well as in enterocin preparations, even after growing the producer cultures in a buffered medium (this excludes the change of pH due to lactic acid production). Similarly, inhibition due to hydrogen peroxide and lactic acid could not be observed, as earlier studied by (Parrot *et al.,* 1990).

Enterococcal growth curve indicating critical growth phase of enterocin production: Enterocin ESF100 production started early during logarithmic phase and the activity reached to maximum in early stationary phase and then remained constant throughout the incubation (Fig. 1). Previously, enterocin RZC5 (from *E. faecium*) reported to be produced in early growth phase (Leroy & De Vl 2002).

Table 3. Effect of different treatments on enterocin ESF100.

Treatments on Enterocin ESF100		
Temperature		
	60ºC for 60 min	R
	80ºC for 40 min	R
	100º C for 30 min	R
	121ºC for 15 min	R
	4ºC for 2 months	R
pH		
	5-8.5	R
Enzymes		
	Catalase	R
	Chymotrypsin	S
	Ficin	R
	Lipase	R
	Lysozyme	R
	Pronase E	S
	Pepsin A	R
	Trypsin	PS
Chloroform vapours		
	30 min	R

R, Resistant; S, Sensitive; PS, partially sensitive

Effect of temperature, pH, enzymes and chloroform: Enterocin ESF100 showed resistance to 60°C for 60min., 80ºC for 40min., 100°C for 30 min., and autoclaving for 15min and remained stable for at least 2 months at 4°C. The pH stability range was found to be between 5-8.5 and was rapidly inactivated in strong alkaline pH conditions. Activity was partially lost after trypsin treatment and completely lost after chymotrypsin and pronase E treatment. No change in bioactivity was observed after catalase, ficin, lipase, lysozyme, and pepsin A treatments. Similarly, no change in size of inhibition zone was observed after chloroform treatment (Table 3).

Lacuna frequency: The percentage of enterocin producing cells in a population was determined by lacuna assay. Lacuna frequency of enterocin ESF100 was determined against two different indicator cultures. It was found that lacuna frequency is different against both the indicator cultures. It is 0.41 against *S. mutans* VSMD (Fig. 2) and 0.51 against *S. aureus* AB211.

Effect of enterocin ESF100 on growing and non-growing cells of *S. mutans* VSMD *and S. aureus* AB211: To test the bactericidal or bacteriolytic mode of action, we investigated the effect of enterocin ESF100 on the viability and lysis of the above mentioned two indicator strains (Fig. 3a, 3b). The addition of enterocin to the growing cells of indicator cultures resulted in rapid and proportional decrease in the viable count during 240 min incubation. Enterocin ESF100 added to non-growing cells also resulted in rapid decrease in the viable count. These results indicated that the enterocin ESF100 exerted a bactericidal mode of action against sensitive cells. Similar findings were reported by (Ohmomo *et al.*, 2000) where enterocin ON-157 produced by *E. faecium* NIAI 157 had bactericidal mode of action.

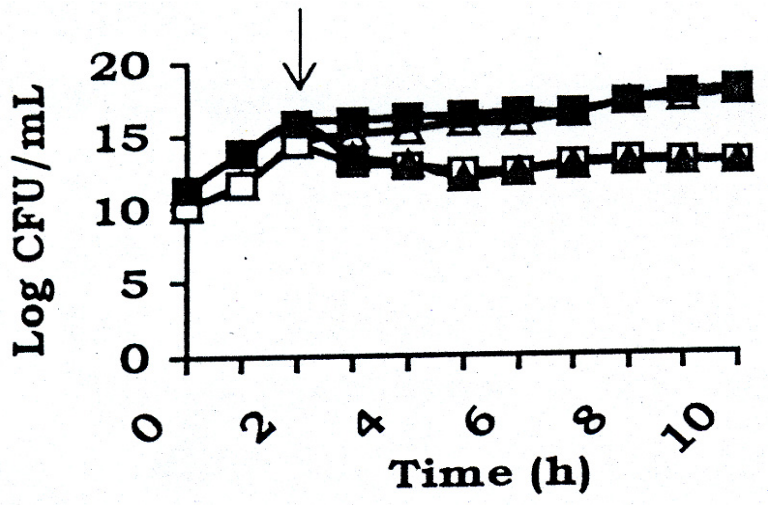


Fig. 3a. Effect of crude enterocin ESF100 on growing cells of *S. mutans* VSMD and *S. aureus* AB211. Enterocin ESF100 was added at 2h after beginning of incubation at 37^0C indicated by a downward arrow. Log of colony forming units (log CFU/mL) of the control (without enterocin ESF100) and enterocin ESF100-treated cells was calculated after every hour.
△ S*treptococcus mutans* VSMD (untreated); □ *Streptococcus mutans* VSMD (treated)
■ *Staphylococcus aureus* AB211 (untreated); ▲ *Staphylococcus aureus* AB211(treated)

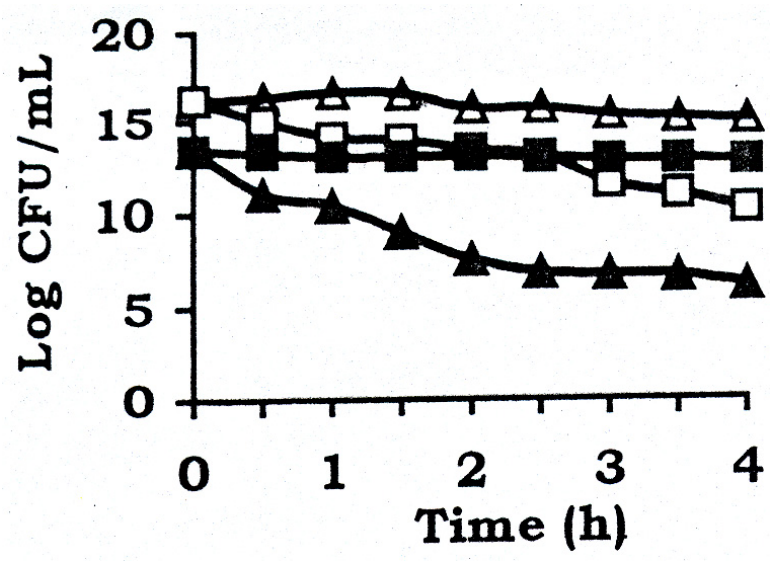


Fig. 3b. Effect of crude enterocin ESF100 on non-growing cells of *S mutans* VSMD and *S aureus* AB211. Log of colony forming units (log CFU/mL) of the control (without enterocin ESF100) and enterocin ESF100-treated cells was calculated after every half an hour.
△ S*treptococcus mutans* VSMD (untreated); □ *Streptococcus mutans* VSMD (treated)
■ *Staphylococcus aureus* AB211(untreated); ▲ *Staphylococcus aureus* AB211 (treated)

Table 4. Effect of curing on drug resistance and enterocin production of *E. faecalis* ESF100 using SDS 2% as curing agent.

Curing agents	Antibiotic resistance pattern (pre-curing)	Antibiotic resistance pattern (post-curing)	Enterocin production (pre-curing)	Enterocin production (post-curing)
2% SDS	FZ	F	+	-

-, No zone of inhibition; +, Zone of inhibition.
F, Fosfomycin; Z, Ceftazidime.

Table 5. Transfer (Conjugation) of drug resistance and enterocin ESF100 production markers using *S. aureus* AB222 as recipient culture.

Culture	Strain	Antibiotic resistance pattern	Enterocin production
Donor (D)	*E. faecalis* ESF100	FZ	+
Recipient (R)	*S. aureus* AB222	F	-
Transconjuant (T)	TSA 222	FZ	+

+, Zone of inhibition; -, No zone of inhibition.
F, Fosfomycin; Z, Ceftazidime.

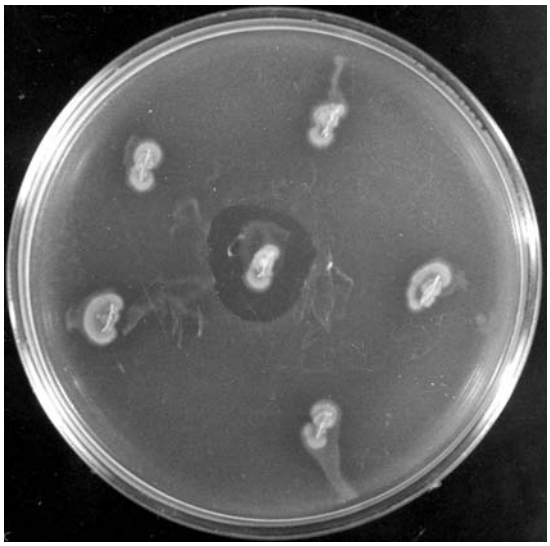


Fig. 4. SDS-mediated curing of *Enterococcus faecalis* ESF100 showing no zone of inhibition by cured (periphery) colonies and zone of inhibition by enterocin producing colonies (center) against *Strepococcus mutans* VSMD.

Plasmid curing, conjugation & agarose gel electrophoresis: Curing experiments were conducted using 2% SDS. It was found that enterocin markers were cured along with the curing of antibiotic ceftazidime suggesting the possibility of the presence of both the genes on one plasmid (Table 4, Fig. 4). The antibiotic and enterocin determinants were stably transferred from *E. faecalis* ESF100 to *S aureus* AB222 (Table 5). It was also noted that bacteriocin genes were transferred along with the ceftazidime resistance genes.

Table 6. Purification of enterocin ESF100 from culture supernatants of *Enterococcus faecalis* ESF100

Sample/step	Volume (mL)	Activity[1] units (AU/mL)	Total activity (AU)	Protein conc. (mg/mL)	Total protein (mg)	Specific[2] activity (AU/mg)	Activity[3] recovered (%)	Fold[4] purification
Culture supernatant	1000	5120	5120,000	22	22000	232.727	100	1
Ammonium sulfate precipitation 80%	100	10000	1000,000	2.4	2400	416.6	19.53	1.7

[1]Activity unit (AU/mL) = Reciprocal of the highest dilution x 1000/volume of enterocin added.

[2]Specific activity (AU/mg) = Total activity of the subsequent purification step/Total protein of the same step.

[3]Recovery (%) = Total activity of subsequent step x 100/Total activity of crude preparation.

[4]Fold purified = Specific activity of subsequent step/Specific activity of crude preparation.

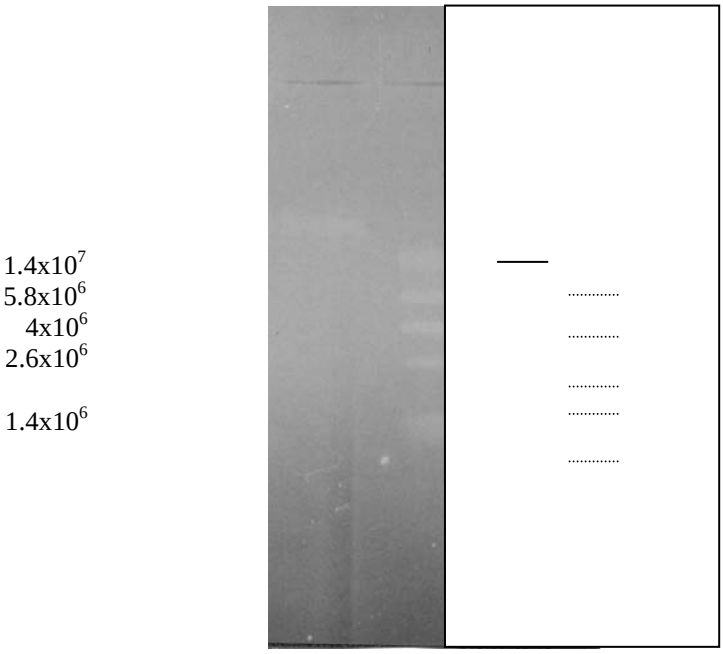


Fig. 5. Plasmid profile of *E. faecalis* ESF100 (enterocin ESF100 producer & cured) after agarose 1% gel electrophoresis. Lane 1, *E. faecalis* ESF100 (enterocin producer), lane 2, molecular weight markers *i.e*. λ *Hind* III digest, lane 3, *E. faecalis* ESF100 (SDS, cured)

Both the genes seem to be present on the same plasmid. Earlier (Ohmomo *et al.,* 2000) reported curing of enterocin plasmid after novobiocin treatment. The plasmid profile analysis clearly exhibits that it harbors plasmid of molecular weight higher than $1.4x10^7$Da whereas, SDS cured *E. faecalis* ESF100 did not show the presence of any plasmid (Fig. 5).

Enterocin ESF100 partial purification: The results of the partial purification of enterocin ESF100 are depicted in (Table 6). All the purification steps were activity directed. Specific activity of crude enterocin ESF100 was 232.7, which increased up to 416.6 (after ammonium sulfate precipitation).

The present study demonstrates that *Enterococcus feacalis* ESF100 produced a bacteriocin-like inhibitory substance i.e. enterocin ESF100 with a broad spectrum of antimicrobial activity directed against both Gram+ve and Gram-ve indicator organisms. Since it can withstand a broad range of temperature and pH, it can be used as useful chemotherapeutic agent. However, further studies are required in this regard.

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