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Partial purification and Characterization of enterocins produced by *E. faecalis* and *E. faecium*

KEYWORDS: Enterococcus faecium, Enterococcus faecalis, enterocin, purification, SDS-PAGE

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ABSTRACT *Bacteriocins produced by lactic acid bacteria have received increasing attention due to their potential use as natural preservatives and as natural substitute for chemical preservatives. In this study, a bacteriocin, produced by Enterococcus faecalis and Enterococcus faecium referred to as enterocin, was partially purified and characterized. Results showed that enterocins was heat stable and stable over a wide pH range. The proteinaceous nature of the antimicrobial compound was ascertained by its sensitivity to many proteolytic enzymes confirming it to be a bacteriocin. Enterocin was partially purified by ammonium sulphate precipitation technique, and the analysis by 16% tris- tricin sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), showed a molecular mass of Enterocins was estimated to be 4.3 to 5.3 kDa.*

Introduction:
Bacteriocins produced by lactic acid bacteria (LAB) are investigated extensively due to their antimicrobial activity against food-borne pathogens[1, 2]. Bacteriocin-producing LAB include lactococci, lactobacilli, pediococci, leuconostoc, and enterococci; the latter are of particular relevance to the current investigation.

Enterococci are ubiquitous lactic acid bacteria isolated from various sources including fermented meat, olives, vegetables and dairy products. They form part of the natural micro flora of the gastrointestinal tract of animals and humans [3, 4]. Two species are common commensal organisms in the intestines of humans, *Enterococcus faecalis* (90-95%) and *Enterococcus faecium* (5-10%).

Enterococcus species produce bacteriocins, known as enterocins. Enterocins are small, ribosomally synthesised, extracellularly released, antibacterial peptides or proteins that display a limited inhibitory spectrum towards other Gram positive bacteria (in particular closely related strains), food-borne pathogens, and spoilage bacteria [5]. Enterocins are microbial active peptides and have developed a great deal of interest as an approach to control food-borne diseases to be used as starter cultures and bio-preservative in various food products. In some cases enterococci are used as probiotics as a result of their protective effects in the gastrointestinal tract [6].

Enterocins usually belong to class II bacteriocins, i.e. they are small and heat stable non-lantibiotics, being stable and able to be produced in the temperature range of 30-37°C. Bacteriocins produced by *Enterococcus species* termed enterocins can be categorized into four classes [7]: Class I (lantibiotic enterocins such as cytolysin (CylLL/ S,[8]); Class II (small, non-lantibiotic enterocins); Class III (cyclic enterocins) such as enterocin AS-48 (EntAS-48,[9]); and Class IV (large proteins), such as enterolysin A (EntL,[10, 11]). Within the Class II, three subclasses can be distinguished: subclass II.1 or pediocin-like bacteriocins, with a strong anti-listerialeVect, possessing the consensus sequence YGNGV in their N-termini, and including, among others, enterocin A (EntA), enterocin P (EntP) and hiracin JM79 (HirJM79); subclass II.2 comprising enterocins synthesized without an N-terminal extension (leader sequence or signal peptide) and/ or requiring two peptides for full antimicrobial activity, such as enterocin L50 (L50A and L50B) [EntL50 (EntL50A and EntL50B)] and enterocin Q (EntQ); and subclass II.3, containing other linear, non-pediocin-type enterocins, such as enterocin B (EntB)[12,13].

Many Enterococci produced antimicrobial peptides as a defense mechanism. Enterocins produced by *Enterococci species* have a wide spectrum of growth inhibitory activity against Gram positive and negative bacteria. Different mechanisms of bacteriocins against target bacteria have been proposed, such as dissipation of proton motive force by pore formation, cell lyses, and interference with degradation and metabolism of macromolecules[14]. As bacteriocins are bioactive peptides and most are cationic at physiological pH. This peptide is highly active against pathogenic bacteria and it displays a dual mode of action at high concentration, it produces localized holes in cell wall and cellular membrane with the leakage of macromolecule such as proteins into external medium and cause death of pathogenic organisms; at lower concentration, it modifies the ion permeability of the cells, dissipating both components of proton motive force [15].

Since *E. faecalis* and *E. faecium* enterocins have been studied, this study was designed to characterize the bacteriocins produced by *E. faecalis* and *E. faecium* for their antimicrobial activity spectrum, evaluating their antimicrobial activity measured in Arbitrary Units (AU /mL), their sensitivity to heat, pH, storage conditions and proteolytic enzymes. Molecular size was also determined.

Material and Method:
Bacterial isolation and identification: Two hundred and fifty consecutive samples for enterococci isolation were included in the study. Enterococci a frequently encounter in dairy products able to produced enterocin, were isolated from dairy products like milk, cheese, etc. Organisms found associated with vaginal tract exhibit better significant inhibitory potential. Clinical samples were collected in sterile broth medium and transferred immediately to laboratory for further processing. Samples were inoculated onto De Man, Rogosa and Sharpe broth for enrichment purpose and incubated at 30°C for 24- 48 hours. The enriched cultures were further analysed for isolation of relevant organisms. The isolation was performed by the routine microbiological isolation procedure and inoculation was performed on selective and differential media viz. Enterococcus confirmatory Agar, De Man, Rogosa and Sharpe Agar, Bile Esculine Agar. All plates were incubated at 30°C for 24- 48 hrs.

Preliminary Screening of Enterocin Producing Isolates: All enterococcal isolates were screened for enterocin production by Agar-well diffusion method [16] against indicator strain *S.*

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aureus. Enterococcal isolates were grown in Brain Heart Infusion broth and incubated at 37°C for 16-18h. For extraction of enterocins, bacterial cells were removed by centrifugation at 10,000X g, for 30min, 4°C. After centrifugation, the supernatant was then adjusted to pH7.0 with 0.1N NaOH. This is cell-free neutralized supernatant, also designated as crude preparation [17]. Brain Heart infusion agar plates were overlaid with 3.0mL soft agar containing 0.1mL (approximately1X-10^6CFU/mL) of the indicator organism. Wells (5mm diameter) were cut and 100µL of cell-free neutralized supernatants of the test organism were poured into each well. Plates were incubated at 37°C for overnight. A clear zone surrounding the bacteriocin producer colonies after growth of the indicator strain was consider as bacteriocin positive. Inhibition zone around the wells were measured and recorded.

Partial Purification of Enterocin:-

Ammonium Sulfate Precipitation: Partial purification of enterocins was carried by using ammonium sulphate precipitation method [18]. The enterocin producer isolates were grown in Brain Heart Infusion broth at 37°C for 16-18hrs. The bacterial cells were removed by centrifugation at 10,000X g, for 30min at 4°C and supernatant was collected. The ammonium sulfate was added slowly to the cell free neutralized supernatant with constant stirring (using magnetic stirrer) till the level of 80% saturation was achieved. The system was held for overnight at 4°C and the precipitates were recovered by centrifugation (10,000X g, for 30 min at 4°C). The resulting pellet was solubilized in 20mM sodium phosphate buffer of pH6.8. The sample thus obtained was designated as crude preparation. The antimicrobial activity of this sample was assayed by using agar-well diffusion method and described in terms of AU/mL. One arbitrary unit (AU) of enterocin was defined as the reciprocal of the serial dilution that showing a clear inhibition zone, multiplied by a factor of 100 (to obtained AU/mL).

Quantitative Determination of Enterocin Activity: The agar well-diffusion method was performed,[19], to determine antimicrobial activity of enterocin. Brain Heart Infusion Agar plates were pre-inoculated with 3.0ml soft agar containing (approximately 1x 10^6 CFU/ mL) of indicator organism and wells of 6 mm diameter were bored in it. Two-fold serial dilutions of cell-free neutralized supernatant in sterile phosphate buffer (pH 0.7) were made and 100µL of each two-fold dilution was pipetted into each well. The plates were incubated at 37°C $\pm$ 2°C for 16-18hrs and diameter of zone of inhibition was measured in mm. The inhibitory strength was expressed as arbitrary unites or activity units/ mL. One arbitrary unit (AU) of enterocin was defined as the reciprocal of the serial dilution that showing a clear inhibition zone, multiplied by a factor of 100 (to obtained AU/mL).

Effect of Physicochemical Treatments on Enterocin Activity:

Thermo Stability Test:- To evaluate the thermal stability, 1ml of enterocin preparations was exposed to different temperatures viz.,60°C for (60 min), 80°C for (40 min), 100°C for (30 min), and 121°C for (15 min). Activity was checked by agar-well diffusion assay[20].

Stability at Different pH Values:- To evaluate the effect of pH on bacteriocin activity, the supernatant pH levels were adjusted between 2.0 and 12.0 using 1 N HCl and 1 N NaOH. The pH stability was assayed at room temperature (25°C) after 1 and 24 hrs of incubation of partially purified enterocin solutions. After incubation, the tested supernatant was re-adjusted to neutral pH and assayed for activity. Untreated samples were used as the control [21].

Effect of Proteolytic Enzymes:- Sensitivity of the enterocin to proteolytic enzyme trypsin, lipase, lysozyme, α-chymotrypsin, Proteinase K and catalase was tested against partially purified enterocin samples. Each enzyme was dissolved in 10 mM sodium phosphate buffer (pH 7.0) and the solutions were added to the bacteriocin solution for a final concentration of 1 mg/ml following incubation at 37 °C for 2 hrs. Untreated samples were used as the control. The residual bacteriocin activity was determined by agar-well diffusion method.

Sensitivity to Chloroform:- To test for chloroform sensitivity the culture supernatant was mixed with an equal volume of chloroform and kept at room temperature for 4 hrs before assessing the antimicrobial activity.

Protein Quantitation:- Protein estimation from crude bacteriocin production was carried out by using Lowry method [22].

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Profiling of Partially Purified Enterocin: Molecular weight of different enterocins were determined from fractions from ammonium sulfate precipitated fraction by performing 16% Tris-Tricin Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [23]. Standard molecular weight marker procured from MBI Fermentas (Mass Ruler ™1500 - 10,000) was used as reference molecular weight marker. Partially purified enterocin solutions obtained from different isolates, were loaded the gel. After electrophorsis, the gel was fixed with a solution containing 15% ethanol and 1% acetic acid. The gel was then washed with distilled water for 4 hrs. The gel was stained with solution containing 0.15% Coomasie brilliant blue R-250 in 40% ethanol and 7% acetic acid to identify the enterocin. The gel was then sequentially washed with phosphate buffered saline for 1.5hrs and subsequently deionized water for 3 hrs.

Results and discussion:

Bacterial isolation and identification:

In this study a total two hundred and fifty samples were collected. Percentage distribution of different samples for isolation of *Enterococcus species* is as shown in Table No.1. Urine samples (38%) followed by Milk (34%) and Cheese (29%) were collected for isolation purpose of *Enterococcus species*. Many other studies also reported isolation of *Enterococcus species*, Franzetti *et al*.,[24] isolated 64 *Enterococcus species* from different sources. Morphological, Physiological and Biochemical identification of Enterococcus isolates was carried out according to the according to standard microbiological techniques. Out of 108 enterococcal isolates 47 (43.51%) from Milk, 35 (32.40%) from Cheese and 26 (24.07%) from Urine were identified. The results revealed that percent isolation of Enterococcal isolates was more in milk followed by cheese and least in urine. The isolated 108 enterococcal isolates were identified as *E. faecalis* 71 (65.74%) and *E. faecium* 37 (34.25%) are depicated in Table No.2. Morandi *et al*.,[25] isolated a total of 68 isolates of enterococci from different North West Italian areas. The isolates were identified as belonging *to E. faecalis*, *E. faecium* and *E. durans*. They concluded from their study that *E. faecalis* and *E. faecium* were the dominant Enterococcal species present in different dairy products; this is also reported by many authors for different cheese varieties (Sarantinopoulos *et al*.,[26]; Achemchem *et al*.,[27]).

Preliminary Screening of Enterocin Producing Enterococci by Agar Well Diffusion Method:

Screening of Enterocin Producing Enterococci determined by Agar Well Diffusion Assay is summarized in Table No. 3. The cell free neutralized supernatants of isolated 108 enterococcal isolates were screened for their enterocinogenic potential against specific indicator microorganism *S. aureus*. It was revealed that, out of 108 enterococcal isolates, 40(37.03%) showed a strong inhibitory activity. Amongst these 40 isolates 27(38.02%) *E. faecium* was found to be and 13(35.13%) was *E. faecalis*.

Selection of Efficient Enterocin Producing Enterococci:

For assessment of antimicrobial activity shown by the efficient enterocin producers, the following isolates were selected. Forty isolates were selected for further study. Eighteen isolates were selected from urine which were further identified as *E. faecium* EMU3, *E. faecalis* EFU5, *E. faecium* EMU7,

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E. faecalis EFU9, *E. faecium* EMU11, *E. faecium* EMU12, *E. faecalis* EFU14, *E. faecium* EMU15, *E. faecalis* EFU16, *E. faecium* EMU19, *E. faecalis* EFU20, *E. faecalis* EFU21, *E. faecalis* EFU23, *E. faecalis* EFU29, *E. faecalis* EFU31, *E. faecalis* EFU33, *E. faecalis* EFU34, *E. faecalis* EFU37. Similarly ten isolates selected from milk were identified as *E. faecalis* EFM38, *E. faecalis* EFM40, *E. faecalis* EFM43 ,*E. faecium* EMM46, *E. faecalis* EFM50 , *E. faecalis* EFM51, *E. faecium* EMM59, *E. faecium* EMM63, *E. faecalis* EFM69, *E. faecalis* EFM70 and twelve isolates were selected from cheese which were identified as *E. faecium* EMC27, *E. faecalis* EFC71, *E. faecalis* EFC72, *E. faecalis* EFC79, *E. faecium* EMC82, *E. faecalis* EFC86, *E. faecalis* EFC87, *E. faecalis* EFC92, *E. faecalis* EFC95, *E. faecalis* EFC98, *E. faecium* EMC101, *E. faecium* EMC105. Their enterocins were designated by adding enterocin to specific isolate number.

Partial Purification of Enterocin:

Out of 40, 12 efficient enterocins was used for further study. The antimicrobial activity in terms of activity units AU/mL was determined. Almost 100 percent antibacterial activity was retained in the precipitates. The antimicrobial activity for cell free neutralized supernatant and partially purified enterocin was found to be 160,000AU/mL and 640,000 AU/mL, respectively. Steps of purification of enterocin from cell free supernatant of *E. faecalis* and *E. faecium* are summarized in table No. 7.

Specific activity for enterocin EMU7 in cell free neutralized supernatant was 44.44 (AU/ mg) which was increased up to 2370.37 (AU/ mg) after ammonium sulphate precipitation. Specific activity after ammonium sulphate precipitation was also found to increase in all other enterocin as compared to cell free supernatant. Specific activity for Enterocin EMU 12, EFU14, EMU15, EFU31, EFM38, EFM 50, EFM 51, EFC 79, EMC 82, EFC92 and EMC 105 were found as 1560.97 (AU/ mg), 1280(AU/ mg), 1684.21(AU/ mg), 1505.88(AU/ mg), 1662.33 (AU/ mg), 1828.57 (AU/ mg), 1391.30(AU/ mg), 1280(AU/ mg), 1422.22(AU/ mg), 1471.26 (AU/ mg) and 1422.22 (AU/ mg) respectively. In our study increased in specific activity after ammonium sulphate precipitation noted is in accordance with other earlier reports of Ahmed *et al.*,[28].

Effect of Physicochemical Treatment on Enterocin Activity:

The effect of temperature treatment on the antibacterial activity of enterocin is summarized in Table No.4. The antimicrobial activity of enterocin remained unaffected when heated at 100°C for 20 min but reduced up to 50% after extended heating at 100°C for 30 min and 121°C for 15min. Similar type of results have been reported for bacteriocin N15 produced by *Enterococcus faecium* N15, which is stable at 100°C but is completely inactivated by autoclaving (Losteinkit *et al.*,[29]). It was observed that all tested enterocin were stable at pH 2 to 8. (Table No. 5). In present study, enterocin produced by the test isolates were screened for their sensitivity (loss of activity) to various enzymes. The inhibitory activity of the enterocin was completely abolished after treatment with the proteolytic enzymes trypsin proteinase K where as activity of enterocin was not lost after α chemotrypsin treatment. However, treatment with lipase, α-glucosidase lysozyme and catalase did not affect the activity of any of tested enterocin. These data clearly showed that the antimicrobial substance is of proteinaceous nature. (Table No.6). Enterocin activities were not affected by lipase, lysozyme, and catalase results are in accordance with the findings of Cocolin *et al.*,[30] and Ghrairi *et al.*,[31].

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Profiling of Partially Purified Enterocin:

Partially purified enterocin fractions were characterized by SDS-PAGE analysis. SDS-PAGE profiling revealed protein bands corresponding to a molecular mass in range of approximately 4.3 to 5.3 kDa. Bands were observered in lane 1, 3, 8, 9, 11 and 12 showed partially purified enterocins from cultures of *E. faecium* EMU7, *E. faecalis* EFU14, *E. faecalis* EFM50, *E. faecalis* EFC79, *E. faecalis* EFC92, *E. faecium* EMC105, respectively, representing molecular mass approximately 5.2kDa while bands observed in lane 4,5,6 and 10 showed bands of partially purified enterocins from cultures of *E. faecium* EMU15, *E. faecalis* EFU31, *E. faecalis* EFM38, *E. faecium* EMC82, having molecular mass approximately 4.3kDa, except for enterocins of *E. faecium* EMU12 and *E. faecalis* EFM 51 which showed slightly different banding pattern possessing approximately 5.3 and 4.1 kDa protein band, respectively, as represented in Plate 15. In the present study, all these molecular weight figures are almost similar to molecular weight observed in earlier studies Ennahar *et al.*, [32]. Dimov *et al.*,[33] demonstrated bacteriocin activity of *E. faecalis*3915. Enterocin3915 produced by *E. faecalis*3915 was characterized by electrophoretic methods after partially purification by ammonium sulphate precipitation method. The results indicated the presence of enterocin of 6.5 kDa molecular weight. Similarly, Line *et al.*,[34] also reported the production bacteriocin E-760. They further characterized enterocin by SDS-PAGE analysis, results revealed 5.5kDa peptide fractions.

Conclusion:

The results of molecular weight, heat stability, and peptide stability at different pH values obtained for *E. faecalis* and *E. faecium* indicate that it produces a bacteriocin that probably belongs to the class IIa. Further studies and more purification steps are encouraged for the practical application of the enterocin of Enterococcus as food preservative as well as in clinical practice.

Table No. 1 :- Percentage Distribution of Different Samples

Sr.No.	Source of sample	% Distribution
1	Milk	34%
2	Cheese	29%
3	Urine	38%

Table No. 2 :- Frequency Distribution of Enterococci in Different Sources

Sr. No.	Identified strains	Urine	%	Milk	%	Cheese	%	No. of isolates	%
1	E. faecalis	17	23.94	28	39.43	26	36.61	71	65.74
2	E. faecium	09	24.32	19	51.35	09	24.32	37	34.25
3	Total	26	24.07	47	43.51	35	32.40	108	

Table No. 3 :- Screening of Enterocin Producing Enterococci by Agar Well Diffusion Method

Sr. No.	Identified strains	No. of enterocin producer strains	Frequency Percentage (%)
1	E. faecalis	13	35.13 %
2	E. faecium	27	38.02 %
3	Total	40	37.03 %

Table No. 4: Effect of Temperature on Enterocin Activity

Sr. No.	Enterocin	Temperature			
		60°C for 40min	80°C for 30min	100°C for 20 min	121°C for 15 min
1	EMU7	+	+	+	-
2	EMU12	+	+	+	-
3	EFU14	+	+	+	-
4	EMU15	+	+	+	-
5	EFU31	+	+	+	-
6	EFM38	+	+	+	-

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7	EFM50	+	+	+	-
8	EFM51	+	+	+	-
9	EFC79	+	+	+	-
10	EMC82	+	+	+	-
11	EFC92	+	+	+	-
12	EMC105	+	+	+	-

(+) activity retained;

(-) activity lost.

Table No.5: Effect of pH on Enterocin Activity

SN	Enterocin	pH									
		2	3	4	5	6	7	8	9	10	11	12
1	EMU7	+	+	+	+	+	+	+	-	-	-	-
2	EMU12	+	+	+	+	+	+	+	-	-	-	-
3	EFU14	+	+	+	+	+	+	+	-	-	-	-
4	EMU15	+	+	+	+	+	+	+	-	-	-	-
5	EFU31	+	+	+	+	+	+	+	-	-	-	-
6	EFM38	+	+	+	+	+	+	+	-	-	-	-
7	EFM50	+	+	+	+	+	+	+	-	-	-	-
8	EFM51	+	+	+	+	+	+	+	-	-	-	-
9	EFC79	+	+	+	+	+	+	+	-	-	-	-
10	EMC82	+	+	+	+	+	+	+	-	-	-	-
11	EFC92	+	+	+	+	+	+	+	-	-	-	-
12	EMC105	+	+	+	+	+	+	+	-	-	-	-

(+) activity retained

(-) activity lost.

Table No. 6: Effect of Enzymes on Enterocin Activity

SN	Enterocin	α- chemotrypsin	Trypsin	Lipase	Proteinase K	Lysozyme	Catalase
1	EMU7	-	-	+	-	+	+
2	EMU12	-	-	+	-	+	+
3	EFU14	-	-	+	-	+	+
4	EMU15	-	-	+	-	+	+
5	EFU31	-	-	+	-	+	+
6	EFM38	-	-	+	-	+	+
7	EFM50	-	-	+	-	+	+
8	EFM51	-	-	+	-	+	+
9	EFC79	-	-	+	-	+	+
10	EMC82	-	-	+	-	+	+
11	EFC92	-	-	+	-	+	+
12	EMC105	-	-	+	-	+	+

(+) activity retained;

(-) activity lost

Table No.7: Partial Purification of Enterocin from Culture Supernatant of E. faecalis and E. faecium

Sr. No.	Sample/ Step	Volume (mL)	Activity [1] Units (AU/ mL)	Total activity (AU)	Protein conce. (mg/ mL)	Total protein (mg)	Specific [1] Activity (AU/ mg)	Activity [3] Recovered	Fold [4] purification
1	*E. faecium* **EMU7**								
	Culture supernatant	1000	160	160,000	3.60	3600	44.44	100	1
	Ammonium sulphate precipitation (80%)	100	ss640	640,000	2.7	270	2370.37	400	53.33
2	*E. faecium* **EMU12**								
	Culture supernatant	1000	160	160,000	3.50	3500	45.71	100	1
	Ammonium sulphate precipitation (80%)	100	640	640,000	4.1	410	1560.97	400	34.14
3	*E. faecalis* **EFU14**								
	Culture supernatant	1000	160	160,000	4.2	4200	38.09	100	1
	Ammonium sulphate precipitation (80%)	100	640	640,000	5	500	1280	400	33.60
4	*E. faecalis* **EMU15**								
	Culture supernatant	1000	160	160,000	3.6	3600	44.44	100	1
	Ammonium sulphate precipitation (80%)	100	640	640,000	3.8	380	1684.21	400	37.89
5	*E. faecalis* **EFU31**								
	Culture supernatant	1000	160	160,000	4.15	4150	38.55	100	1
	Ammonium sulphate precipitation (80%)	100	640	640,000	4.25	425	1505.88	400	39.06

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6	*E. faecalis* **EFM38**								
	Culture supernatant	1000	160	160,000	3.65	3650	43.83	100	1
	Ammonium sulphate precipitation (80%)	100	640	640,000	3.85	385	1662.33	400	37.92
7	*E. faecalis* **EFM50**								
	Culture supernatant	1000	160	160,000	4.10	4100	39.02	100	1
	Ammonium sulphate precipitation (80%)	100	640	640,000	3.50	350	1828.57	400	46.86
Sr. No.	Sample/ Step	Volume (mL)	Activity [1] Units (AU/ mL)	Total activity (AU)	Protein conce. (mg/ mL)	Total protein (mg)	Specific [1] Activity (AU/ mg)	Activity [3] Recovered	Fold [4] purification
8	*E. faecalis* **EFM51**								
	Culture supernatant	1000	160	160,000	4.55	4550	35.16	100	1
	Ammonium sulphate precipitation (80%)	100	640	640,000	4.60	460	1391.30	400	39.57
9	*E. faecalis* **EFC79**								
	Culture supernatant	1000	160	160,000	4.60	4600	34.78	100	1
	Ammonium sulphate precipitation (80%)	100	640	640,000	5	500	1280	400	36.80
10	*E. faecium* **EMC82**								
	Culture supernatant	1000	160	160,000	4.65	4650	34.40	100	1
	Ammonium sulphate precipitation (80%)	100	640	640,000	4.50	450	1422.22	400	41.34
11	*E. faecalis* **EFC92**								
	Culture supernatant	1000	160	160,000	4.75	4750	33.68	100	1
	Ammonium sulphate precipitation (80%)	100	640	640,000	4.35	435	1471.26	400	43.68
12	*E. faecium* **EMC105**								
	Culture supernatant	1000	160	160,000	4.30	4300	37.20	100	1
	Ammonium sulphate precipitation (80%)	100	640	640,000	4.50	450	1422.22	400	38.23

[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 Purification = Specific activity of subsequent step / Specific activity of crude preparation.

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