

Biochemical characterization and identification of enterocins produced by *E. hirae* And *E. faecalis*

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ABSTRACT

The present study was an endeavour to partially purify and characterize enterocins produced by *E.hirae* and *E.faecalis* from UTI patients. Maximum enterococcal isolates were obtained from females of 10–20years age group and compared with males of same age group. Enterocin was partially purified by ammonium sulphate precipitation technique followed by dialysis and analysis by SDS-PAGE revealed molecular mass of enterocin to be 4.5–5.5 kDa approximately. Partially purified enterocin was heat and pH stable and sensitive to proteolytic enzymes, thus proving it to be of proteinaceous nature and was therefore characterized as enterocin. Such bacteriocin have broad field of application including both food industry and medical sector.

KEY WORDS: *ENTEROCOCCUS HIRAE* ,DIALYSIS, *E.FAECALIS*, ENTEROCIN, SDS-PAGE.

INTRODUCTION

For years, Enterococci have been considered as harmless inhabitants of the gastrointestinal tracts of humans and animals. The genus *Enterococcus* is a heterogenous group of bacteria, which includes 20 different species. The interest on Enterococci is raised in the last decades mainly for 2 important characteristics; they are considered as infection agents especially in immune compromised hosts and they are used as useful probiotics and starter cultures in various fermented food (Maki and Agger 1998; Leroy *et al.*, 2003; Manolopoulou *et al.*, 2003).

Enterococci produce bacteriocins, Known as enterocins which are proteinaceous compounds produced by bacteria that exhibit a bactericidal or bacteriostatic mode of action against sensitive gram positive and gram negative bacteria. *Enterococci* are part of the normal intestinal microflora and may become opportunistic pathogens in individuals with in serious diseases whose immune systems are compromised. *E.faecium* and *E.faecalis* are known to produce enterocins which possess heat stability and antilisterial activity, (Wilaipun *et al.*, 2004, Enan, 2006, Sawa *et al.*, 2009, Jamaly *et al.*, 2010).

Urinary Tract Infection (UTI) is a common infection prevalent among various age groups. Although UTI is

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common in women it can affect both gender and all age groups. Untreated UTI in a long run can lead to many complications such as pyelonephritis and renal damage. Although Enterococci is commonly isolated from UTI it is less often speciated. Speciation and antibiogram of enterococci is gaining relevance because of emerging antimicrobial resistance. The selection and identification of an enterocin produced by an *Enterococcal* strain isolated from urine is of interest because it can be used as probiotic bacterium inhibit other bacterial pathogens (Gamal *et al.*, 2006 and Civamani *et al.*, 2014).

Many enterococci produce enterocins which are short peptide as a defense mechanism. Bacteriocins (enterocins) and are used bioactive peptides and most are cationic at physiological pH. This peptide is highly active against pathogenic bacteria and it plays a dual mode of action at high concentration, it produces localized holes in cell wall and cellular membrane which leads to leakage of macromolecule such as protein into external medium and cause death of pathogenic organism; at lower concentration; it modifies the ion permeability of the cells, dissipating both components of proton motive force (Minahk *et al.*, 2004). Enterocins have developed a great deal of interest as an approach to control food borne diseases to be used as starter culture and biopreservative in various food products. In some cases, enterocin are used as probiotics as a result of their protective effects in GIT (Khan *et al.* 2016)

Thus, considering all this, the study was undertaken to characterize enterocins from *E. hirae* & *E. faecalis* and to evaluate its inhibitory activity against indicator strain. The antimicrobial activity was measured in Arbitrary units (AU/ml). Molecular size was also determined along with its activity against heat, pH, proteolytic enzymes and storage conditions.

MATERIAL AND METHODS

STUDY DESIGN

A descriptive study was performed at Biochemistry Dept., Shri Shivaji College of Arts, Commerce and Science, Akola. The urine specimens were collected from government hospital, private hospital and pathology laboratory. Patients with complaints of fever, burning micturition and pain lower abdomen were included in the study. The urine samples were collected and processed in the laboratory by standard methods. The name, age, sex and date of onset of symptoms was noted. On isolation of enterococci, the organism was speciated and its activity was checked using different parameters. All the data obtained was recorded using MS Excel Software. The statistical analysis included statistics like frequency percentage, mean and standard deviation.

BACTERIAL IDENTIFICATION

Total one hundred and eight urine samples were collected for Enterococcal isolation. 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 hrs. The enriched cultures were then analyzed for isolation of relevant organism. The isolation was performed by the routine microbiological procedure and inoculation was performed on selective and differential media viz Enterococcus Confirmatory Agar, De Man Rogosa, Sharpe Agar and Bile Esculin Agar. All plates were then incubated at 30°C for 24-48 hrs.

SCREENING OF ENTEROCIN PRODUCING ISOLATES

All enterococcal isolates were screened for enterocin production by Agar-well diffusion method against indicator strain *S. 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 at 4°C. After centrifugation, the supernatant was adjusted to pH 7.0 with 0.1N NaOH. This is cell-free neutralized supernatant, also designated as crude preparation. Brain Heart infusion agar plates were overlaid with 3.0mL soft agar containing 0.1mL (approximately 1X-10⁶CFU/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 considered as bacteriocin positive. Inhibition zone around the wells were measured and recorded.

PARTIAL PURIFICATION OF ENTEROCIN

Ammonium Sulfate Precipitation and Dialysis

Partial purification of enterocins was carried out by using ammonium sulphate precipitation method (Harris *et al.*, 1989) 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 pellet obtained was then suspended in 20 mM sodium phosphate buffer in dialysis bag (Dialysis tubing D0405 Sigma Aldrich) and was dialysed for over-

night at 4°C with constant stirring. 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, to determine antimicrobial activity of enterocin. Brain Heart Infusion Agar plates were pre-inoculated with 3.0ml soft agar containing (approximately 1×10^6 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 100 μ L of each two-fold dilution was pipetted into each well. The plates were incubated at 37°C + 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 (30min), and 121°C for (15 min). Activity was checked by agar well diffusion assay (Iqbal *et al.*, 1999).

Stability at Different pH Values: Enterocin activity was also checked by placing it on wide range of pH. 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.

Effect of Proteolytic Enzymes: Sensitivity of the enterocin to proteolytic enzymes trypsin, α -chymotrypsin, lipase, lysozyme 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 enterocin 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 enterocin

activity was determined by agar-well diffusion method (Ahmad *et al.*, 2003). **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 enterocin preparation was carried out by using Lowry method (Lowry *et al.*, 1951).

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 poly-acrylamide gel electrophoresis (SDS-PAGE). Standard molecular weight marker procured from GeNei TM (GeNei™ 1500 - 10,000) was used as reference molecular weight marker. Partially purified enterocin solutions obtained from different isolates, were loaded on the gel. After electrophoresis, 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% Coomassie 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 1hr and subsequently deionized water for 3 hrs. (Parbal 1988)

RESULTS AND DISCUSSION

Bacterial Isolation and Identification: In the present study, total 108 urine samples were collected from UTI patients. Mean values in Table 1 states that maximum isolates obtained were from females as females are more prone to urinary tract infections. The figures related to standard deviation shows that heterogeneity is more in males of all age groups as compared to females with respect to their dietary habits, physical workout and mode of living. Morphological, physiological and biochemical identification of enterococcal isolates were carried out according to Standard microbiological technique. Out of 108 samples 40 enterococcal isolates were obtained and identified as *E. hirae* 14 (35%) and *E. faecalis* 26 (65%) Results were expressed in mean (standard deviation)

Max isolates (45.37%) were seen in 10-20yrs age group followed by 21-40 yrs age group (34.25%). 62.03% represents that max isolated from females as compared to 41 (37.96%) from males.

Preliminary screening of enterocin producing enterococci

Screening of enterocin producing enterococci was done by agar well diffusion method is summarized in table no.2

Age groups(yrs)	Male	Female	Total	Standard Deviation
10-20 yrs	20 (18.51%)	29 (26.85%)	49 (45.37%)	2.84
20-40 yrs	13(12.03%)	24(22.22%)	37 (34.25%)	5.13
40-60 yrs	8 (7.40%)	14(12.96%)	22 (20.37%)	3.56
Total	41(37.96%)	67(62.03%)	108 (100%)	
Standard Deviation	(13.16)	(12.50)	(12.71)	

Identified strains	No.of producer strains/ No.of tested strains	Frequency Percentage (%)
<i>E.hirae</i>	8/14	57.14
<i>E. faecalis</i>	18/26	69.23
Total	26/40	65

The obtained isolates were screened for their enterocinogenic potential against specific indicator strain of *S.aureus*. It was observed that out of 108 samples 40 enterococcal isolates were obtained showing strong inhibitory activity. Amongst these 40 isolates, 8 (57.14%)

out of 14 were found to be *E.hirae* and 18 (69.23 %) out of 26 of *E.faecalis* were found to be efficient producer of enterocin. For the assessment of antimicrobial activity shown by efficient enterocin producers six isolates were selected for further study. These were desig-

Sr. No.	Sample / Step	Volume (ml)	Activity units (AU/ml)	Total activity (AU)	Protein Conc. (mg/ml)	Total Protein (mg)	Specific activity	Activity recovered	Fold purification
1)	<i>E.hirae</i> EHE 8								
	Culture Supernatant	1000	160	160000	3.80	3800	42.10	100	1
	Ammonium sulphate precipitation 80%	100	640	640000	2.8	280	2285.71	400	54.29
2	<i>E. faecalis</i> EFE 9								
	Culture Supernatant	1000	160	160000	4.5	4550	35.16	100	1
	Ammonium sulphate precipitation 80%	100	640	640000	4.6	460	1391.30	400	39.57
3	<i>E.hirae</i> EHE 10								
	Culture Supernatant	1000	160	160000	3.6	3600	44.44	100	1
	Ammonium sulphate precipitation 80%	100	640	640000	4.2	420	1523.80	400	34.28
4	<i>E. faecalis</i> EFE 15								
	Culture Supernatant	1000	160	160000	4.7	4700	34.04	100	1
	Ammonium sulphate precipitation 80%	100	640	640000	4.3	430	1488.37	400	43.72
5	<i>E.hirae</i> EHE 18								
	Culture Supernatant	1000	160	160000	3.5	3500	45.07	100	1
	Ammonium sulphate precipitation 80%	100	640	640000	4.1	410	1560.97	400	34.63
6	<i>E.faecalis</i> EFE 21								
	Culture Supernatant	1000	160	160000	4.3	4300	37.20	100	1
	Ammonium sulphate precipitation 80%	100	640	640000	5.0	500	1280.00	400	34.40

Table 4: Effect of different temperature on Enterocin activity

Sr. No.	Enterocin	Temperature treatment			
		60°C for 60 min	80°C for 40 min	100°C for 30 min	121°C for 15 min
1	EHE 8	+	+	+	-
2	EFE 9	+	+	+	-
3	EHE 10	+	+	+	-
4	EFE 15	+	+	+	-
5	EHE 18	+	+	+	-
6	EFE 21	+	+	+	-

(+) - Activity retained (-) - Activity lost

Table 5: Effect of different pH on Enterocin activity

Sr. No.	Enterocin	pH treatment										
		2	3	4	5	6	7	8	9	10	11	12
1	EHE 8	+	+	+	+	+	+	+	-	-	-	-
2	EFE 9	+	+	+	+	+	+	+	-	-	-	-
3	EHE 10	+	+	+	+	+	+	+	-	-	-	-
4	EFE 15	+	+	+	+	+	+	+	-	-	-	-
5	EHE 18	+	+	+	+	+	+	+	-	-	-	-
6	EFE 21	+	+	+	+	+	+	+	-	-	-	-

(+) - Activity retained (-) - Activity lost

nated as *E.hirae* EHE8, *E.faecalis* EFE9 *E.hirae* EHE10 *E.faecalis* EFE15 *E.hirae* EHE18 *E.faecalis* EFE21. Their enterocins were named by adding enterocin to specific isolate number. One of the most important characteristic of enterocin is evaluation of susceptibility for different antibiotics. The obtained isolates were susceptible to commonly used antibiotics like ampicillin, amoxicillin while resistant to norfloxacin only. Thus stating its use as efficient and safe for clinico-medical sector (Khan et.al.2016)

Partial Purification of Enterocin: The partial purification of enterocin from cell free supernatant of *E.hirae*

and *E.faecalis* was done as mentioned in table no.3. The antibacterial activity was determined in terms of activity units AU/ml. The antibacterial activity for cell free neutralized supernatant and partially purified enterocin was found to be 160000 AU/ml and 640,000AU/ml respectively. It was seen that the specific activity of enterocin EHE8 in cell free supernatant was 42.10(AU/mg) which was increased upto 2285.71 (AU/mg) after ammonium sulphate precipitation and dialysis. Specific activity of all remaining enterocins were found as EFE9 1391.30(AU/mg), EHE10 1523.80(AU/mg), EFE15 1488.37(AU/mg), EHE18 1560.97(AU/mg) EFE21 1280(AU/mg). Thus, spe-

Table 6: Effect of different Enzymes on Enterocin activity

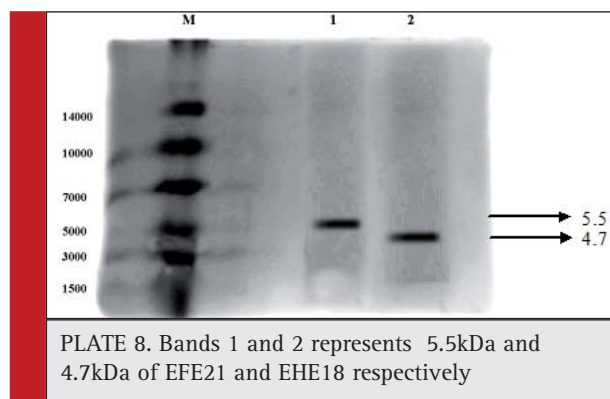
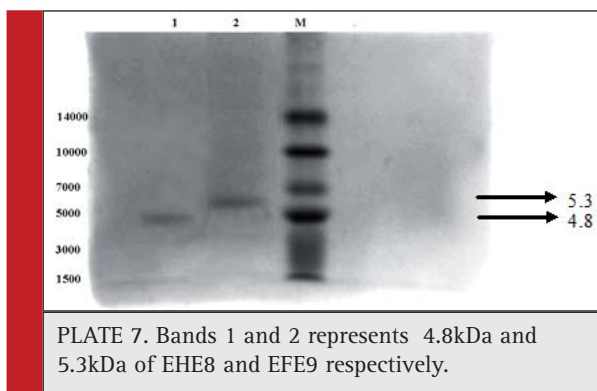
Sr. No.	Enterocin	Catalase	Trypsin	α -chymotrypsin	Lipase (pepsin A)	Lysozyme
1	EHE 8	+	-	-	+	+
2	EFE 9	+	-	-	+	+
3	EHE 10	+	-	-	+	+
4	EFE 15	+	-	-	+	+
5	EHE 18	+	-	-	+	+
6	EFE 21	+	-	-	+	+

(+) - Activity retained (-) - Activity lost
 Abbreviations: Enterocin from respective isolates,
 1.EHE 8- *E.hirae* EHE8 , 2.EFE 9- *E.faecalis* EFE 9, 3.EHE 10- *E.hirae* EHE10
 4.EFE 15-*E.faecalis* EFE 15, 5.EHE 18- *E.hirae* EHE18, 6 EFE 21-*E.faecalis* EFE 21

cific activity after ammonium sulphate precipitation and dialysis was found to be increase in all crude enterocins as compared to cell free supernatant.

Effect of physicochemical treatment on Enterocin activity: The effect of thermal treatment is as summarized in table no 4. All crude enterocins were stable following boiling for 30mins. The antimicrobial activity remained unaffected when heated at 100° C for 30min but activity gets reduced or lost beyond 121° C for 15min. All the tested enterocins were stable to wide range of pH from 2-8 as shown in table 5. Table 6 shows the effect of various enzymes on inhibitory activity of enterocins. It demonstrated sensitivity to proteolytic enzymes trypsin and α chymotrypsin while treatment with catalase, lipase and lysozyme did not affect the activity of enterocin. Thus, the obtained data suggests that the loss of enterocin activity to proteolytic enzyme is due to the fact that enterocin contained an essential proteinaceous component while stability in presence of both lipase and lysozyme indicates that it lacked lipid or carbohydrate moieties. Similar enterocin activities to different physicochemical parameters were also seen in Cocolin *et al.*, (2007) Our results are also in accordance with the findings of Ahmad *et al.*, (2003) and Dezwaan *et al.* (2007).

SDS-PAGE Profiling of Partially Purified Enterocin: The crude enterocin obtained were further characterized by SDS PAGE analysis. The results of partial purification of obtained enterocin fraction from *E.hirae* and *E.faecalis* are depicted in plate 7 and plate 8. SDS-PAGE profiling revealed protein bands having molecular mass in range of approximately 4.5 to 5.5kDa. Bands were observed in lane 1 and 2 showed partially purified enterocin from cultures of *E.hirae* EHE8 and *E.faecalis* EFE 9 representing molecular mass approximately 4.8kDa and 5.3kDa in plate 7. Bands 1 and 2 in plate 8 represents molecular mass of bands from partially purified enterocins from cultures *E.faecalis* EFE21 and *E.hirae* EHE 18 as 5.5kDa and 4.7kDa respectively. In the present study the molecular weight observed is similar to those obtained



inearlier studies. Line *et al.*, also characterized enterocin by SDS-PAGE analysis which reveals 5.5kDa enterocin fraction. Similarly, Park *et al.*, identified partially purified bacteriocin produced by *E.faecium* JCM 5804^T, enterocin A and enterocin B of molecular mass 4.5kDa approximately.

CONCLUSION

The current study thus describes the biochemical characterization of enterocin from *E.hirae* and *E.faecalis* from urine samples. It was found that enterocin was heat stable and stable over wide range pH2-8 sensitive to proteolytic agents trypsin and α chymotrypsin. Molecular size ranges 4.5-5.5kDa which states that obtained enterocin may belong to class IIa bacteriocin. Several enterocins have been characterized to date, many of which are produced by Enterococci which have potential to cover a very broad field of applications including both the food industry and clinico-medical sector.

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