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Partial purification and characterization of enterocin SN11 produced by *Enterococcus Hirae* from animal feed samples

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ABSTRACT

Enterocin are a wide group of bacteriocin produced by enterococcus spp, many enterocin show bactericidal activity against pathogens such as gram positive and gram negative microorganisms. A bacteriocin producing strains were isolated from the animal feed samples of maize, rice husk, spelt, oil cake, peanuts. They have maximum production of bacteriocin was optimized at 37°C for 36hrs and at a pH of 7.0. The partial separation of enterocin was performed by precipitation with ammonium sulphate and subsequent ion exchange chromatography followed high pressure liquid chromatography. In the current work an enterocin termed as SN11 was been identified with the molecular weight of enterocin SN11 >5kDa, and it belongs class II bacteriocin of enteriocin. In the present study it is focused that purified enterocin SN11is used for food bio preservation, to prevent urinary infection, vaginal and gastro intestinal infection causing bacterium. It displayed excellent antibacterial activity against six different pathogens. Hence, the study concludes that enterocin SN11 consist of peptides, which intracts into the target cell membrane and leads to pores formation and finally induces cell death.

Keywords: *Enterococcus hirae*, antimicrobial activity, enterocin, bio-preservative, pharmaceutical.

INTRODUCTION

The genus *Enterococcus* species of gram positive, non-spore forming, negative of oxidase and catalase negative, and facultative anaerobic, this belonging to the heterogenous group of latic acid bacteria (LAB). It as single cocci some time pair with short chains, optimal growth at 35°C, even though can grow MRS broth in the presence of 6.5% NaCl, pH 9.6 at temperatures from 10-45° C.

Bacteriocin also called as proteins are ribosomally synthesized bacterial peptide with has antimicrobial activity, but not constantly, those strongly associated to the producing bacteria, moreover it can active beside the *Listeria monocytogenes*, *Staphylococcus aureus*, and *Bacillus cereus* those are belongs as gram positive food borne pathogens. Close with the LAB as well as *enterococci* produce heterogenous group of bacteriocin, their potential application in food, pharmaceuticals, nutraceuticals, veterinary and human medicine [1, 2, 3, 4].Mostly *Enterococcus* are implicated in food spoilages of food, nosocomial infection and leads to antibiotic resistant [5, 6, 7]. On the aminoacid sequences similarity if and its inhibitory spectrum of some bacteriocins produced by *Enterococcus* species (the enterocin) it is like the group of pediocin [8, 9]. According to the Klaenhammer *et al* bacteriocin classified four groups based on their protein molecular weight and amino acids sequences. Class I (lantibiotics) consists of thermostable peptides molecular range of <5 kDa, of lanthionine dehydroalanine,

dehydrobuthyrine, and β-methyl-lanthionine with the structure.of dehydro-amino acids and thioether amino acids. In Class II (non-lanthibiotics) does not contain lanthionine, it consists of thermo stable peptides active against *listeria* species their molecular weight of <10kDa. There are three subgroups; Class IIa / pediocin like peptide active against *listeria* with an N-terminal consensus sequence of Tyr-Gly-Asn-Gly-Val-Xaa-Cys (YGNGVxC); Class IIb (Two peptide of complementary action required for the bacteriocin activation) and Class IIc (contained active thiol group peptides it require reduced cysteine for trigger the activation). Class III as thermolabile proteins it consists of thermo-sensitive proteins (>30 kDa). The Class IV consisted of peptide complex whose activity requires lipid or carbohydrate molecules together with the protein fraction [10, 11, 12].

All bacteriocin well known synthesized as pre-peptide with an N-terminal leader sequence of outside the cell membrane. Bacteriocin (enterocins) synthesized by the general secretory pathway / ATP-binding cassette transport system [13, 14, 15, 16]. Double-glycine motif was present in bacteriocin leader peptide it serves as a signal for processing and secretion [17]. The leader peptide is usually positively charged and has a hydrophobic core and with a cleavage region [18, 19, 20]. This leader peptide is synthesized by a signal peptidase during translocation across the cytoplasmic membrane. The antimicrobial peptides has many biological function such as blocking of membrane protein synthesis, inhibition of DNA synthesis, antiviral properties, and antitumor effects as well as induction of apoptosis or cytotoxicity of tumor cells [21, 22], due to this confirmation antimicrobial peptides have been considered as potential therapeutic drugs [23, 24].Nowadays bacteriocins like nisin, have been used for prevention of growth of cancer cells. Nisin is not toxic to animals, is safe for human consumption, and was approved for human use by the WHO in 1969 and by the FDA in 1988.

In this study, we characterized the enterocin SN11 which is a new enterocin like pediocin family of bacteriocin, termed enterocin SN11 identified in *Enterococcus hirae*.

EXPERIMENTAL SECTION

Procurement and maintenance of cultures

The bacterial species like *Staphyolcoccus aureus, Streptococcus pneumoniae* MTCC-655, *E.coli* spp MTCC-1583, *Klebsiella pneumoniae* MTCC-39, *Shigella* spp MTCC-2957, *Camphylobacter* spp and *Salmonella typhimurium* MTCC 98 was procured from IMTECH, Chandigarh, India. The strains were revived and maintained in nutrient agar medium pH6.8 at 37ºC for 24hrs. All *enterococcus* spp strains were grown in MRS broth and culture medium (Himedia laboratory Mumbai, India), at 37°C for 24 hrs. Mueller Hinton agar and all the other regents such as ammonium sulphate, bovine serum albumin were purchased from Himedia laboratory Mumbai, India and CM sepharose was obtained from Amersham Pharmacia.

Screening of bacterial strains

The sample cultures were isolated from different brands of animal feeds Maize (M1, M2, M3 and M4), Rice husk (R1, R2, R3 and R4), Spelt (S1, S2, S3 and S4), Oil Cake (Oc1, Oc2, Oc3 and Oc4) and Peanuts (P1, P2, P3 and P4). The feed materials were dissolved in sterile Normal saline (pH 7.0), furthermore 10 fold serial diluted samples was surface plated with respective selective medium (MRS). The plates were incubated at 37 ºC for 24 hrs. The *Enterococci spp* positive was identified by grams staining, biochemical and sugar fermentation patterns as per Bergey's Manual of Systematic Bacteriology [25].

Phylogenetic analysis of 16S rRNA sequencing

The primary structure results was aligned manually using with Genetic Data Environment (GDE) software (Smith, 1992). And the aligned primary structure was compared with previously described 16S rRNA gene sequences from *Enterococcus* spp and relatives, as retrieved from GenBank. 16S rRNA gene sequencing was carried out to find the species level.

Enterocin production and extraction

After passing of the quality control testes like staining, biochemical and sugar fermentation experiments the isolated bacterial strain was subsequently seeded (1%) the inoculum in MRS broth (pH7.0) for overnight under aerobic condition at 37 ºC for 48 hrs. After 48 hrs the bacterial cells mass were removed from MRS broth by 8000×g for 15 min under 4ºC, the cell bacterial pellet pH was adjusted 6.5 using sterile 1N NaOH for the exclusion of organic acid mediated antimicrobial effects. In continuation it was further saturated with 80% ammonium sulphate by overnight stirred under 4ºC and following the cooling centrifugation at 10,000 rpm for 30minutes, in additional the pellet was resuspended in 25 mL of 0.05 M potassium phosphate buffer (pH7.0) for agar well diffusion methods described by Kang and Lee [32].

Bacteriocin assay
The saturated bacterial pellet was further sterilized with 0.22 µm pore size membrane filtration the subjected aliquots (50 µl) were loaded in 4-mm-diameter wells of MRS agar plates containing previously seeded with indicator bacterial strain and further incubated at 37^0C for 18 to 24 hrs, after incubation the diameters of the inhibition zones were measured in millimetre.

Antibiotic susceptibility testing
Antibiotic susceptibility studies were performed by disc diffusion method. Ampicillin, vancomycin, teicoplanin, rifampicin, erythromycin, tetracycline, chloromphenicol, ciprofloxacin, quinupristin-dalfopristin, nitrofurantoin were used for the study. High level amino glycoside resistance was determined by disc diffusion. The disc diffusion screening method was performed on Mueller Hinton agar. These discs transferred to plates prepared with agar base plate are incubated at 37^0C upright position. The inhibition zone was evaluated after overnight incubation.

Optimization of growth conditions for production of enterocin
The bacteriocin production levels was analysed in different incubation time, temperature and pH the experiments was conducted with 100 mL MRS broth in 250 ml of sterile Erlenmeyer flasks, the bacteria was inoculated and overnight incubated at different temperatures ranges like 35, 37, 39 and 41 ºC respectively, in case of pH experiments the ranges of pH like 6.6, 6.8, 7.0, 7.2 and 7.4 and various incubation times like 12, 24, 36 and 48 hrs respectively. The tested samples were collected after 48 hrs (exclusion of incubation time effect) and analysed enterocin production by the spectrometric reading at 480nm [26].

Inoculum concentration of *Enterococcus hirae*
Enterocin production level in different inoculum concentration, evaluated temperature at 37^0C, was performed with 100 mL of MRS broth in 250 mL of Erlenmeyer flasks that were inoculated with different concentration of freshly prepared seed inoculums like 0.5, 1.0, 1.5, and 2.0mL respectively, after incubation Samples were collected enterocin production were measured at 480nm.

Enterocin purification by ion exchange chromatography
After the dialysis of ammonium sulphate saturated bacterial cell free protein was subjected in cation exchange chromatography for the partial enterocin purification by the following sequences 20 mL of the dialysate onto 10 mL bed volume of CM Sepharose (bed height- 6.5 cm). Fast Flow chromatography column (110 mm to 20 mm) was equilibrated with phosphate buffer pH 7.0 at ambient temperature as per the methodology of Jagannathan et al (2015) [27] and Shivanandappa et al (2015) [34]. The protein fractions were eluted by replenishing with linear salt gradients like 0.1 to 0.7 M NaCl, [28] in phosphate buffer (pH 7.0) total volume 40 mL with the of 8 mL/hrs of flow rate. The fraction volume was 2 mL. After elution, the fractions were subjected for purity by 260/280 nm and the antibacterial activity through agar well diffusion methods. 50 µl of the fractions were subjected to purity as y the method of Glasel *et al* 1995 [29]. The culture medium was used as a negative control in case of antibacterial activity test, as per the higher OD values of the eluted fractions was selected for anti bacterial analysis when compare with the standard strains likes *Staphyolcoccus aureus, Streptococcus pneumoniae* MTCC-655, *E.coli* MTCC-1583, *Klebsiella pneumoniae* MTCC-39, *Shigella* MTCC-2957, *Camphylobacter spp* and *Salmonella typhimurium* MTCC 98.

High pressure liquid chromatography
The selected, pooled fractions from ion exchange column of E. *hirae* proteins was further applied to C18 reverse-phase column (Polaris C8-A; 250-mm as length, the internal diameter was 10-mm and the pore size was 180-Å, the particle size was 5-m for high-pressure liquid chromatography (HPLC) system, the elution pattern was made with H_2O acetonitrile containing 0.1% TFA as a gradient and the flow rate of 2 mL/min the resulted peaks was were detected at 280 nm by using a false array detector.

Molecular weight determination by tricine SDS-PAGE
HPLC subjected /purified samples (10µl) purity were further assessed by tricine SDS-PAGE along with low molecular weight marker protein was performed as per [30], the post run analysis the tricine SDS-PAGE gel was stained with silver staining, further it was distained twice in 10% acetic acid.

RESULTS AND DISCUSSION

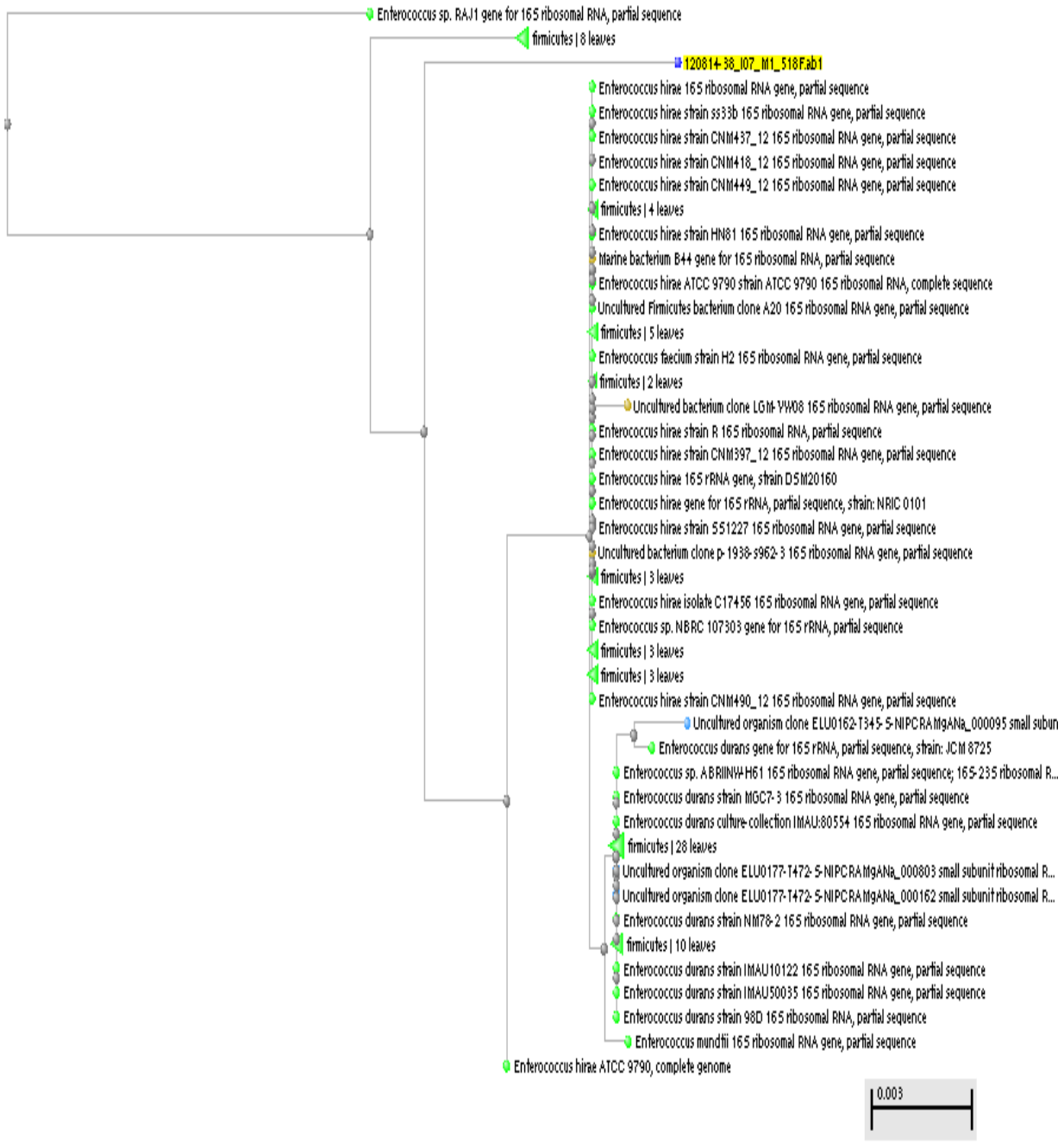
In this research study five various animal samples like maize, rice husk, spelt oil cake and peanuts and inoculated with MRS medium for the isolation of *Enterococcus* spp and it was confirmed via morphological, cultural, fermentation and biochemical tests and found that gram positive, facultative anaerobic and carbohydrate

fermentation and the mean cultural pH range was between 6.5±7.0 and the biochemical confirmation test results were catalase and oxidase negative.

Identification of strains and molecular phylogeny

The confirmed *Enterococcus* strains were further persisted to 16S rRNA sequence analysis, the proximal portion of the gene was confirmed with existing available reference strain in database, in this study the ribotype 16S rRNA sequence was carried out at sophisticated instrumentation laboratory, Indian Institute Technology-Chennai, India and the strain was identified as *Enterococcus* hirae and submitted to Gene bank with the accession number GI: 618928025

Phylogenetic Tree of *Enterococcus hirae* showing 99% sequence similarity



Antagonistic activity of the cell free supernatant

The supernatant of *Enterococcus* spp was subjected to antagonistic activity with Gram positive and negative bacterial strains and the results was presented in Table 1, the following standard bacterial species was used like

Staphyolcoccus aureus, Streptococcus pneumoniae MTCC-655, *E.coli* MTCC -1583, *Klebsiella pneumonia* MTCC-39, *Shigella* MTCC-2957, *Camphylobater* spp and *Salmonella typhimurimum* MTCC-98 and *Camphylobater* spp shown maximum activity with *Enterococcus* spp , in case of minimal activity was found with *Staphyolcoccus aureus, Streptococcus pneumoiae* MTCC-655, and *E.coli*.

Table 1 *Enterococcus* spp isolated from different animal feed samples

S. No	Animal feed samples	Code
1.	Maize	M1, M2, M3 & M4
2.	Rice husk	R1, R2, R3 & R4
3.	Spelt	S1, S2, S3& S4
4.	Oil cake	Oc1, Oc2, Oc3& Oc4
5.	Peanuts	P1, P2, P3& P4

Detection of antibiotic resistance of *Enterococcus* species

The five isolated bacterial strains were subjected for antibiotic resistance study, there are 8 antibiotics disc were used like N30, G30, DO30, MET5, C30, VA30, FIF5 and CE30, all the five isolates (*E. hirae, E. faecium, E. avium, E. mundtii* and *E. duram*). All five isolates were susceptible to the entire antibiotics (Table 2) in addition *Enterococcus* species like *E. hirae* , *E. avium and E. durum* was found maximum resistance against all tested antibiotics.

Table 2 Inhibition of various indicator organisms by Enterocin produced by *Enterococcus* spp

Enterococci spp	*Staphyolcoccus aureus*	*Streptococcus pneumonia*	*E.coli*	*Klebsiella pneumonia*	*Shigella spp*	*Salmonella typhimurium*	*Camphyl spp*
M1	++	+	**N**	++	+	+	+
M2	+	++	++	++	++	++	++
M3	++	**N**	+	+	+	+	+
M4	+	+	+	+	+	+	+
R1	**N**	++	+	+	+	+	+
R2	++			++	+	+	+
R3	+	++	++	++	++	++	++
R4	+	+	+	+	++	+	+
S1	+	++	++	++	++	++	++
S2	+	**N**	**N**		++	+	+
S3	++	+	+	++	+	+	+
S4	**N**	++	+	+	+	+	+
Oc1		++	+		+	+	+
Oc2	++	+	+	++	+	+	+
O3	++	++	+	+	+	+	+
O4	+	++	++	++	++	++	++
P1	+	++	++	++	++	++	++
P2	++	**N**	+	++	+	+	+
P3	+	+	**N**		++	+	+
P4	**N**	++	++	++	++	++	++

Symbols: +: minimum inhibition activity (within 1 ± 7 mm)
Symbols: ++: maximum inhibition activity (within 7 ± 12 mm)
Symbols: N: No inhibition activity

Influence of growth condition for the Enterocin production

On the early stationary growth phase at 18th hrs of *Enterococcus*, the enterocin secretion was started and the production was started at the late log phase into the culture medium, the secretion level was decreased at stationary phase. During the optimal temperature analysis for the secretion of enterocin was observed at 37ºC from 6 hours onwards until 36 hr at 41ºC, the level was in lower. In the optimal pH analysis the enterocin level at pH was found at 0.480,0.931,1.182,3.720,2.981 and 2.812 at 6.12,12,18,24,30 and 36 hrs respectively it reveals that pH 7.0 is more optimal when compared with different pH ranges like 6.6,6.8,7.2 and 7.4, the pH plays a vital role for the secretion of enterocin. In case of time ranges, maximum enterocin level was observed at 36 hrs and minimum enterocin production was recorded at 12 hrs was detailed in given table 3, 4 and 5. The seed inoculum concentration played important role in cell growth as well as enterocin production, furthermore high productions of strain recorded were at inoculum concentration of 2.0 ml and the minimal enterocin production was observed at 0.5ml (Table.6)

Table 3 Antibiotic sensitivity of different *Enterococcus* spp

Enterococci spp	N30	G30	DO30	MET5	C30	VA30	FIF5	CE30
E. hirae	S	R	S	R	R	R	R	R
E. faecium	R	R	S	S	R	R	S	R
E. avium	R	R	R	S	R	R	S	R
E. mundti	S	S	R	R	R	S	R	R
E. duram	R	R	S	R	R	R	S	R

S – Sensitive; R – Resistant.

Table 4 Enterocin produced by *Enterococcus hirae* (Different Temperature Rate)

Temperature	Sample collection hours					
	6h	12h	18h	24h	30h	36h
35^0C	0.231	0.531	1.234	2.434	2.434	1.983
37^0C	0.481	0.824	1.631	3.372	3.241	2.750
39^0C	0.410	0.830	1.596	3.121	2.989	2.750
41^0C	0.352	0.724	1.420	2.843	2.891	2.762

Table 5 Enterocin produced by *Enterococcus hirae* (Different pH Range)

pH	Sample collection hours					
	6h	12h	18h	24h	30h	36h
6.6	0.231	0.481	0.981	1.831	1.931	1.856
6.8	0.389	0.781	1.381	2.213	2.343	2,246
7.0	0.480	0.931	1.821	3.720	2.981	2.812
7.2	0.492	1.021	2.123	3.014	2.581	2.481
7.4	0.385	0.753	1.483	2.340	2.240	2.150

Table 6 Enterocin produced by *Enterococcus hirae* (Different time Range)

Hours	Sample collection hours					
	30^0 C	37^0 C	44^0C	51^0 C	58^0 C	65^0 C
12	0.241	0.213	0.191	0.180	0.140	0.125
24	0.256	0.295	0.214	0.198	0.175	0.160
36	0.267	0.760	0.561	0.350	0.291	0.171
48	0.261	0.321	0.231	0.320	0.289	0.254

Purification and characterization of enterocin

In order to purify the enterocin the bacterial cell membrane was removed by centrifugation, subsequently the bacterial pellet was saturated with 80% of ammonium sulphate precipitation, and the residual ammonium was removed by dialysis with appropriate dialysis membrane, each and every experiments the protein concentration as well as the antimicrobial activity was analysed and found within the limit also without higher protein losses. The dialysate further subjected to ion exchange chromatography the eluted fractions protein purity as well as the concentration was analysed by A261/A280 (Fig 1), higher protein concentrated fractions like fraction 4 to Fraction 11 total 8 fractions was confirmed its antimicrobial activity with standard bacterial species and found passed as well as significantly increased total activity and same time the biological activity was also increased by agar well diffusion method it reveals the purity of the enterocin the resulted figures like Fig 2 (A,B,C,D,E F and G).

High performance liquid chromatography

When the ion exchange performed selected fractions were pooled and further subjected in HPLC the sample was made to run for 10 min and the input volume was 10 µl and recorded to 250 nm -320 nm, during the process methanol was used as the solvent system .The enterocin were detected by false array detector at 280 nm, and the peak (267231-height, RT-2.097) was obtained, and it was compared with standard reference enterocin peaks and found significantly (Table7 and Fig 3).

Molecular weight determination in Tricine SDS

After the tricine SDS-PAGE of the enterocin, a single band was around 5kDa evidently indicates the protein purity (Fig 4).

Table 7 *Enterococcus hirae* Inoculum Concentration

Inoculums	Sample collection hours					
	6h	12h	18h	24h	30h	36h
0.5	0.320	0.640	1.210	2.381	2.521	2.321
1.0	0.387	0.698	1.310	2.420	2.420	2.127
1.5	0.498	0.821	1.521	2.814	2.814	2.891
2.0	0.581	1.021	2.121	3.041	3.141	2.981

Table 8 HPLC for *E. hirae*

S.No	RT	Area	%Area	Height
1.	2.097	3482513	86.50	267231
2.	3.215	19756	0.49	1902
3.	4.118	523811	13.01	16658

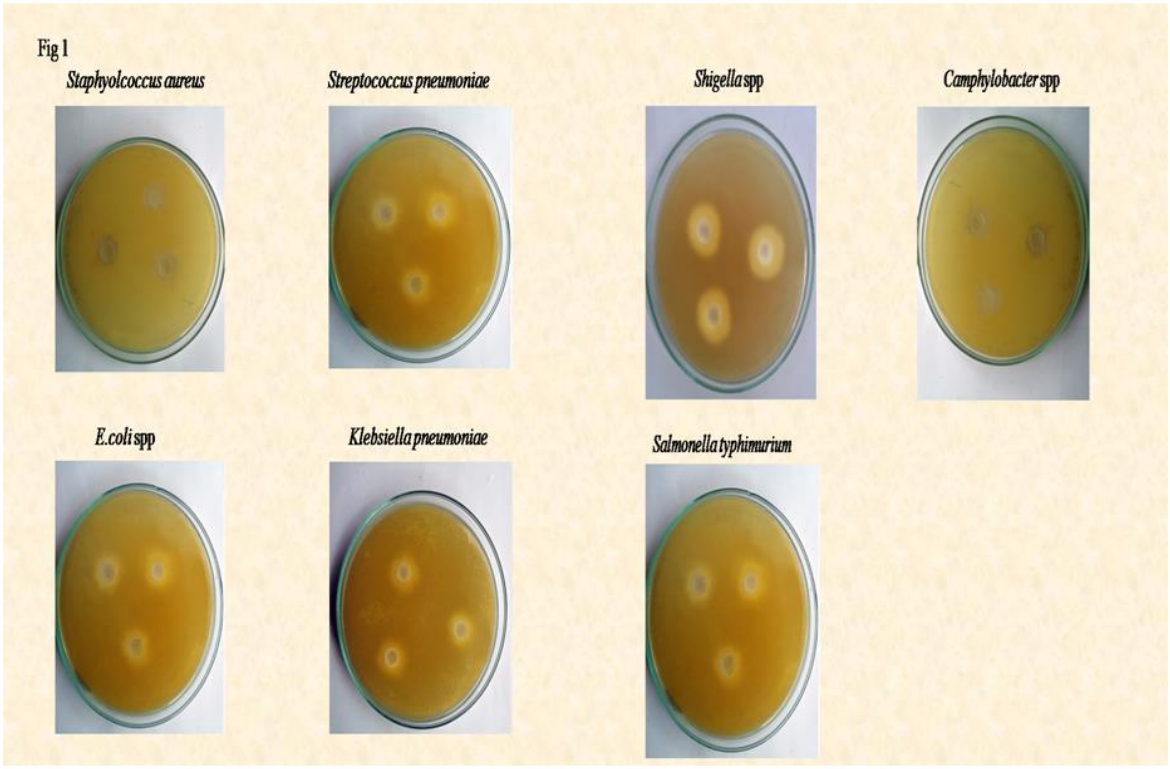


Fig 1 Antagonistic activity of enterocin SN11 against pathogenic microorganisms

Fig. 2a

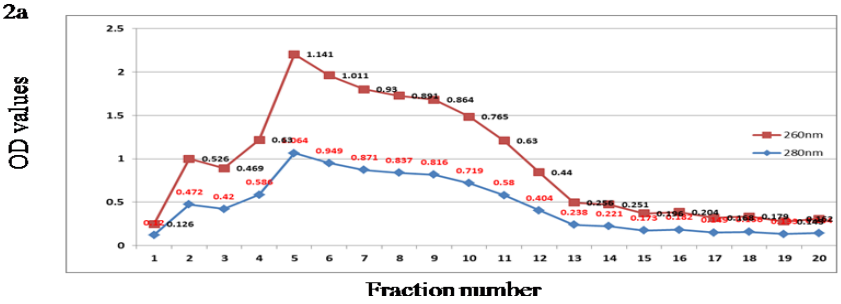


Fig. 2a.Ion exchange chromatography purification of enterocin SN11

Fig. 2b

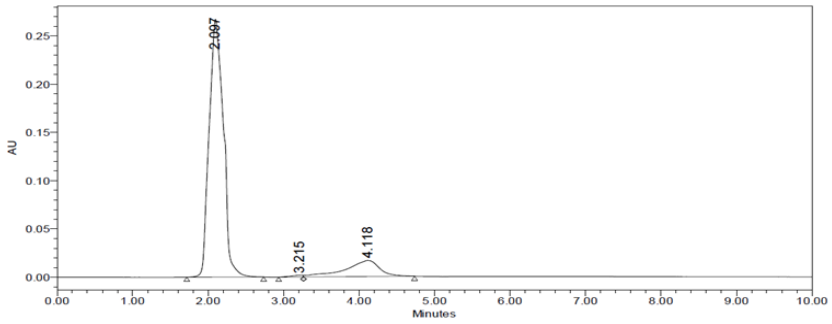


Fig. 2b.High pressure liquid chromatographic analysis of enterocin SN11

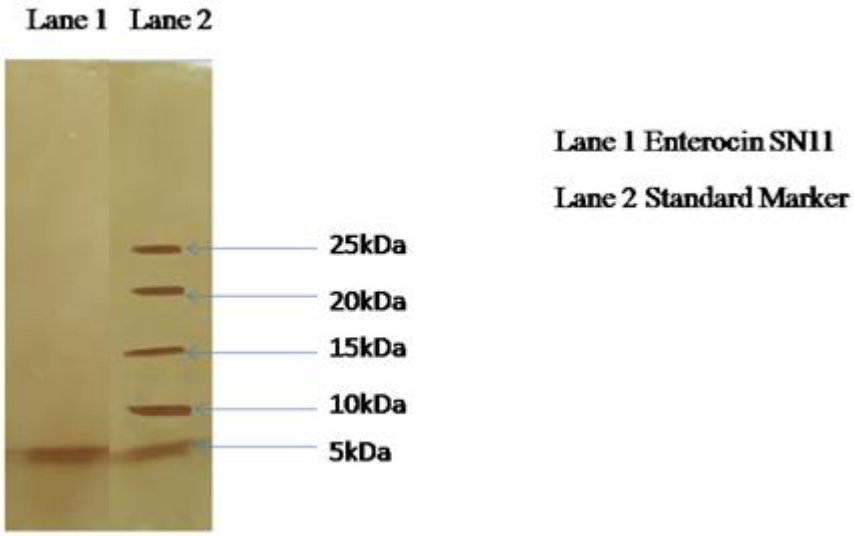


Fig. 3.Tricine SDS-page analysis of purified enterocin SN11

Probiotic bacteria *Enterococcus* spp strains were found to be antimicrobial agent with great potential for use in food industry, attributed by production of enterocin. In the present study, the enterocin produces were enriched in MRS medium. The isolated organism was identified as *E. hirae, E. faecium, E. avium, E. mundtii* and *E. duram* by biochemical test. Further species level of *E.hirae* was confined by 16S rRNA sequencing. The isolate strain was confirmed to be enterocin the activity of the strain through the agar well diffusion methods. *E.hirae* produced high enterocin, thereby for the mass production of enterocin for food industry and therapeutic application *E.hirae* can be recommended. In the present study, the stability of enterocin was applied under extreme pH, temperature, time duration and different inoculums concentration, the enterocin showed higher production at pH-7.0. The enterocin production was found highest at 37^0C, and at the higher concentration of inoculum it was found early stage enterocin production higher. Further purification of enterocin was done by cationic ion exchange chromatography and the collected fractions were tested for antimicrobial property. The enterocin showed significant inhibition against food borne pathogenic and intestinal microorganisms such as *Staphyolcoccus aureus, Streptococcus pneumoniae* MTCC-655, *E.coli* spp MTCC-1583, *Klebsiella pneumoniae* MTCC-39, *Shigella* spp MTCC-2957, *Camphylobacter* spp and *Salmonella typhimurium* MTCC 98. Results obtained purification after indicated that enterocin SN11 was highly hydrophobic in nature. The activity increased several purification processes parallel with an increase of specific activity. The spectrum of partially purified enterocin SN11 was the same to that observed with agar well diffusion, but with strong activity against pathogenic microorganisms. The result showed the presence of enterocin component identified by high pressure liquid chromatography with according to the reference Esther Izquierdo *et al*., 2008 [31].

The data from the present study suggests to the enterocin SN11 to be a class II a bacteriocin as the molecular weight is >5KDa and is stable at different pH and temperature. This is the first characterization of a class IIa enterocin produced by animal feed strains of *E.hirae.* These results are more encouraging as a drug for pharmaceutical, food preservation, urinary infection, vaginal and gastro intestinal tracts.

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