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Isolation And Identification Of Enterocin Isolated From Various Reservoirs And Their Antimicrobial Potential.

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Abstract: *The genus Enterococcus consists of gram positive, facultative anaerobic organisms. The aim of the present was to purify and therapeutic potentialities of enterocin from various species of Vancomycin resistant Enterococcus (VRE) sps. A method for isolation protein was developed Antimicrobial assay was studied by well diffusion assay. It was observed that isolated enterocin showed potent antibacterial activity. Maximum activity was observed in Streptococcus grisveus (18mm) at 80 µg/ml. Maximum activity was observed at 80 µg/ml against Candida albicans. Mainly newly expressed proteins were found to remain from the original mixtures based on SDS-PAGE. The results showed that this protein exhibited great potential of antimicrobial activities. The present results confirm that this protein can be used as drug, in various therapeutic ventures.*

Key words: - *Enterococcus faecalis; Enterococcus facium; Enterocin ; Vancomycin resistant Enterococcus (VRE) ; Antimicrobial ; SDS- PAGE.*

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I. Introduction

The genus Enterococcus belongs to category gram positive and are facultative anaerobic colonies which bears ovoid shape and also visualized as smear in pairs, short chains, along with unicellular like other streptococci species. They are found in every habitat of ecological diversity and can grow and stick with in insensitive climatic conditions and also found in the fecal microbiota of many organisms . The chronic pathogenicity produced by these strains are main cause of urinary infection followed by intra abdominal and pelvic infection. They are responsible for UTI, endocarditis, neonatal sepsis, surgical wound infection, bacteramia, super infections etc.

E. faecalis is the leading organism for (80-90%) of infection. *Enterococcal endocarditis* generally found in children and not often in infants (Sood *et al*, 2008). Enterocin is a kind of protein synthesized by bacteria. It resists the growth of other kinds of bacteria. It is really a proteinaceous toxin that is formed as a small molecule by bacteria. It reduces the viability of bacterial strains that are analogous or closely associated. Entereocin are ribosomally synthesized antimicrobial peptides produced by microorganisms categorized in different taxas. The synthesis of small antibiotic peptides is a common defense strategy against bacteria that is displayed not only by microorganisms, but also by animals and plants (Oscáriz and Pisabarro, 2001). Recently they have been reported and these enterocins displayed strong inhibitory action against microorganisms.

Thus in the present investigation an attempt has been made for isolation and purification of enterocin from selected species and their antimicrobial potential.

II. Materials and Methods

Collection of bacterial strains-193 strains of Vancomycin resistant *Enterococcus faecalis* and *Enterococcus faecium* isolated from urine, blood, pus, and faeces of the patients of the SMS Hospital, Jaipur. These isolates were subjected to Gram's staining and catalase test and were tested for their ability to ferment a variety of carbohydrates and for their capability of growth at 10°C and 45°C in media containing 6.5% NaCl at pH 9.6. After this initial characterization, the strains were further characterized using molecular methods (Vrinda Ramakrishnan et al, 2012). Some indicator strains such as *Pseudomonas aeruginosa* (Multi drug resistant) isolated from patient of burn ward and from wound infections, *E.coli* from blood and urine, *Staphylococcus aureus* from skin infection, *Bacillus* spp. from stool, *Streptococcus* from pus and sputum. All isolates were then identified by morphological and biochemical characterization using traditional methods. All

these Strains were maintained in BHI (Brain Heart Infusion Agar) broth. All indicator strains were kept frozen in BHI with 20% glycerol at -20^{o}C.

Isolation of enterocin

Initially Vancomycin resistant *Enterococcus spp.* colonies were grow in BHI broth for 24 hours. After growth of the strain it was centrifuged at 15000 rpm for 20 minutes at 4^{o}C. Then supernatant was collected and neutralized with 1N NaOH. Supernatant was passed through the membrane filter (.22µm) after that Ammonium Sulphate was added up the saturation with constant stirring at room temperature. It was again centrifuged at 15000 rpm for 20 minutes at 4^{o}C. Supernatant was discarded and pallet was dissolved in 100mM Sodium Phosphate buffer. The sample was dialyzed overnight at room temperature. Next day it was again dialyzed with Poly- ethylene Glycol (Ahmed et al., 2004.)

Effect of temperature and pH range on enterocin- Thermal stability of enterocin was checked by exposing it to different temperature like room temperature and -20^{o}C, -10^{o}C, 0^{o}C, 40^{o}C, 60^{o}C 80^{o}C, 100^{o}C and autoclaving for 30 minutes. The pH range of enterocin was adjusted from 3-12 with 10mM HCl or 10mM NaOH. The pH of enterocin was adjusted to 7.0 with phosphate buffer for activity (Nemade and Musaddiq, 2013 ; Sarika et al., 2010).

Antibacterial Activity against human pathogens- The agar well-diffusion method was performed to determine the antibacterial activity of enterocin (Perez *et al*, 1990) Nutrient agar medium was seeded with overnight culture of indicator strain and was poured into a Petri dish. Wells (8-mmdiameter) were cut with sterilized cork-borer from the indicator-seeded agar and 20µg/ml to 80 µg /ml of enterocin was poured in to each well. After diffusion of enterocin in to agar medium plates, were incubated upside down for 24 hours at 37 °C. After 24 Hrs. zones of inhibition of the indicator species around the wells were measured in mm.

Antifungal activity of enterocin

The agar well-diffusion method was performed to determine the antifungal activity of enterocin (Bonjar et al, 2005) Potato dextrose agar medium was seeded with 48 hours culture of indicator strain and was poured into a Petri dish. Wells (8-mmdiameter) were cut with sterilized cork-borer from the indicator-seeded agar and 20µl, 40 µl, 60 µl, 80 µl of enterocin was poured in to each well. After diffusion of enterocin in to agar medium plates, were incubated upside down for 48 hours at 37 °C. After 48 Hrs. zones of inhibition of the indicator species around the wells were measured in mm.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Profiling of Enterocin
SDS-PAGE analysis

SDS–PAGE was performed according to (Laemmli, 1970) using 15% polyacrylamide gel, run at 100 V. Proteins standard was of medium range 16 –209 kDa was used as molecular weight markers and visualized by staining with coomassie brilliant blue G-250.

III. Results

The enterocin producing strain previously isolated from urine, blood**,** pus, and faeces of the patients of the SMS Hospital, Jaipur, India. These strains were identified as *Enterococcus faecalis* and *Enterococcus faecium* by the biochemical, cultural and morphological features such as colony morphology on MRS agar was grayish, round, with flat edges and elevated from center, Gram positive cocci with cells arranged uniformly or in groups. When various biochemical tests were performed it was found that the strain was negative when assayed by catalase and oxidase . *Enterococcus faecalis* produced acid from Manitol, Rafinose, Sorbose but *Enterococcus faecium* produced acid from L-Arabinose Sorbitol Manitol. The final pH in glucose broth was 4±2, grew in 6.5% NaCl broth at 9.6 *p*H. It possessed non-hemolytic on blood agar plates having composition of 5-7% of sterile sheep blood.

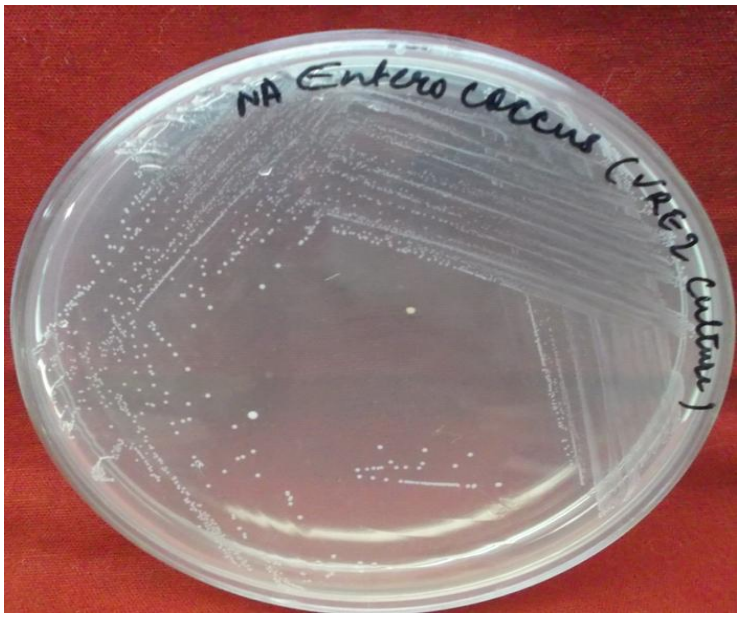


Fig-1 Growth of *Enterococcus* on Solid media.

Antimicrobial activity

It was observed that isolated enterocin showed potent antibacterial activity. Maximum activity was observed in *Streptococcus grisveus* (18mm) at 80 µg/ml while in other strains activity was at par at 20 µg/ml at all concentrations activity was potent **(Table 1)**

Table No.1 Antibacterial activity of Enterocin

Enterocin (in µg/ml)	***Pseudomonas aeruginosa*** IZ (in mm)	***Streptococcus grisveus*** IZ (in mm)	***E.coli*** IZ (in mm)	***Staphylococcus aureus*** IZ (in mm)	***Bacillus subtilis*** IZ (in mm)
20	10±0.23	10±0.23	10±0.23	11±0.25	12±0.28
40	13±0.34	13±0.34	12±0.28	13±0.34	13±0.34
60	15±0.41	15±0.41	13±0.34	14±0.36	16±0.48
80	17±0.56	18±0.62	14±0.36	16±0.48	18±0.62

IZ-inhibition zone in mm

Table No.2 Antifungal activity of Enterocin

Enterocin (in µg/ml)	***Candida albicans*** IZ (in mm)	***Penicillium funiculosum*** IZ (in mm)	***Fusarium oxysporium*** IZ (in mm)	***Trichoderma reesei*** IZ (in mm)
20	-	-	-	-
40	10±0.23	-	-	-
60	10±0.23	-	-	-
80	12±0.28	10±0.23	-	11±0.25

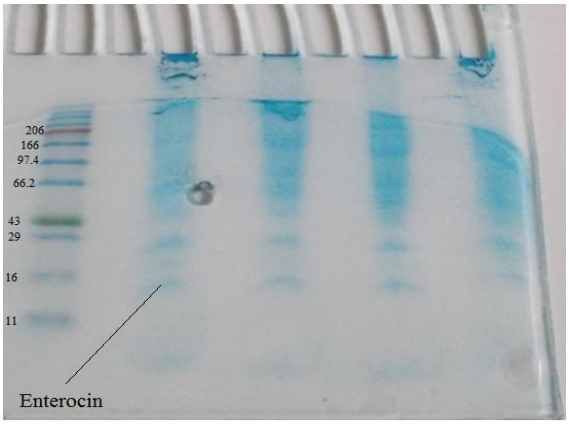
IZ-inhibition zone in mm , **(-)No zone of inhibition**

However when isolated enterocin was tested against various fungal strains it was observed that some strains were found to be resistant. Maximum activity was observed at 80 µg/ml against *Candida albicans*. Further we observed that at 40 and 60 µg/ml. the activity was at par**.** *Fusarium oxysporium* was found to be totally resistant as the sample did not possessed any activity at various concentrations **(Table 2).**

SDS-PAGE

The isolated protein from these bacteria showed the highest purity of 2.500 based on absorbance of 0.026 at 280 nm and 0.065 at 615 nm. The molecular weight was determined by relative mobility. As can be seen in Fig. 2, the molecular weight of enterocin was approximately16.5 kDa which was almost similar in other lanes.

M L_1 L_2 L_3 L_4



M = Marker (11, 16 29, 43, 66.2, 97.4, 166 and 206 kDa)
L1, L2, L3 L4 (Lane1, Lane2, lane3, lane4) = sample (enterocin)
Fig. 2

IV. Discussion

Enterocin are having numerous applications in food technology for its preservation duration storage (Ghrairi et al., 2012), which bears barrier against many pathogens (Van Heel et al.,2011). It also has major role in pharmaceutical and medical industry to combat with various cancers. These are consumed as food additives which are safe to use as they are required in metabolic process in the body of individual of particular habitat. They also consumed as natural food additives due to the as they are consumed during synthesis process of yogurts, Portuguese fermented meat (Todorov et al., 2014). Since they possess relative profusion and their conflict to environmental factors, they have been proposed as an indicator bacteria for hygiene quality, as well as for antimicrobial resistance in food and water (Boehm and Sassoubre, 2014). They have evolved as important in treating of harmful pathogens which are directly associated with health issues (Arias and Murray, 2012), as they are fundamentally resistant and shows resistivity against commercial available antibiotics and are able to acquire drug resistance by using various manipulations of recombinant DNA technology. There are various bacteriocins isolated from LAB in meat and dairy products have been reported (Deraz et al. 2005), bacteriocin KCA2386 (8.1 kDa) produced by *Lactococcus lactis* (Ko & Ahn 2000), plantaricin 35d (4.5 kDa) produced by *L. plantarum* 35d (Messi et al. 2001), bacteriocin ST44AM (6.5 kDa) *from Pediococcus pentosaceus* ST44AM (Todorov & Dicks 2009), bacteriocin AMA-K (2.9kDa) from *L. plantarum* AMA-K , bacteriocin ST414BZ (3.7 kDa) from *L. plantarum* ST414BZ (Todorov & Dicks 2010), sakacin C2 (5.5 kDa) from L. sakei C2 (Gao et al. 2010).

Conflict of interest

The authors declare that they do not have any conflict of interest.

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