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Isolation, Partial Purification and Biochemical Characterization of Enterocin Producing Enterococci

KEYWORDS: UTI, Enterocin, Tripticase Soy Agar, Enterococci

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ABSTRACT *Bacteriocins are bacterial proteins or peptides that inhibit strains and species that are uaually but not always closely related to the producing bacteria.Enterocins are novel bacteriocin produced by enterococcus species. Many enterococcus species from various ecosystems were characterized as antagonists of broad range of pathogens. Broad spectrum activities against prominent pathogen make it an issue of medical interest. The ability to produce such biocompound may play a role in providing an ecological advantage on non bacteriocin producer strains. Thus, 40 strains of enterococci were isolated from urine samples of UTI patients. These strains were identified to species E.faecalis and E.hirae. Enterocin activity of all the 40 tested isolate was checked against indicator strain on Tripticase Soy Agar by agar well diffusion assay. From these 26 isolates displayed strong inhibitory activity against indicator strain. The purified enterocin was found to be active against all Gram positive and Gram negative bacteria.*

Introduction
Enterococci are natural inhabitants of gastrointestinal of humans and animals,but are also found in other anatomical sites including the vagina and oral cavity and in plants and insects. (Elisa Bittencourt de Marques et.al 2004, Devriese et al 1992)*Enterococci*, an indigenous flora of the intestinal tract, oral cavity and the genitourinary tract of humans and animals are known to be relatively avirulent in healthy individuals but have become important opportunistic pathogens especially in hospitalized patients. They belong to group D Streptococci as characterized by Lancefield in 1938 whose taxonomy has changed considerably in last few years.Recent years have witnessed increased interest in *Enterococci* not only because of their ability to cause serious infections like endocarditis, bacteremia, intra abdominal and urinary tract infections but also because of their increasing resistance to many antimicrobial agents.This emphasizes the need for their identification from clinical specimens and also differentiate them from other group D Streptococci which are generally more sensitive to the antimicrobial agents.(Desai PJ et.al. 2001).

A large number of micro organisms produce a variety of compounds which demonstrate antimicrobial property.One such group of these compounds is the enterocin,which consists of relatively small bactericidal peptides.Enterocin are defined as compound produced by bacteria that have biologically active protein moiety and bactericidal action.These enterocins are novel bacteriocin produced by *Enterococcus sps*. The ability to produce such biocompound may play a role in providing an ecological advantage on non bacteriocin producer strains.These enterocins are active against various pathogenic such as Listeria,Clostridium,Staphylococcus and E.coli.(Foulquie M.R.,Moreno *et.al.*)

Enterococci are gram positive homofermentative lactic acid bacteria that can tolerate a wide variety of growth conditions like temperatue of 10^0 to 45^0 c,hypotonic, hypertonic,acidic or alkaline environments. Sodium azide and concentrated bile salts which inhibit or kill most micro organism are tolerated by *Enterococci*. As facultative organism, *Enterococci* grow under reduced or oxygenated conditions. Enterococcal bacteriocins i.e. enterocin have gained attention in recent years because bacteriocin producing strains can be isolated with ease from several fermented food and also from various clinical specimens like urine,skin swab,pus and blood. Apart from humans and animals this bacteria can also be found in the soil and water while some strains of E. faecalis can cause opportunistic and hospital acquired infections in humans, many other strains have been used for a variety of beneficial purposes by both nature and man.(Desai PJ et.al. 2001).

Thus, keeping in mind wide applications and occurrence of *Enterococci*,the work has been done to isolate and partially purify the enterocin from *enterococcus* species.

Material and Methods:
The enterocin producing strains used in this study were *E.faecalis and E.hirae* .For this study, 68 urine samples were taken from the urinary tract infected patients which was used as producer of enterocin. All the clinical specimen i.e.urine samples were collected from various pathology laboratories of Akola and processed in Dept.of Biochemistry, Shri Shivaji College of Arts,Commerce and Science Akola.

Bacterial Strains and Media:
The enterocin producing strains were selected in this study. For isolation of *Enterococcus* species, the clinical specimen i.e.urine was collected from the urinary tract infected patients which was used as producer of enterocin. All the collected samples were inoculated on MRS broth for enrichment at 37^0 c for 24hrs.Enriched culture was surface plated on Enterococcal selective medium i.e. Bile Esculin Agar and Enterococcus Confirmatory Agar. Plates were than incubated at 30^0c for 24hrs.Typical colonies obtained on agar were screened for Gram character and subjected to various tests like enzyme test, esculin test and tolerance to 6.5% NaCl 40% bile tolerance etc.

Isolation and partial purification of Enterocin:
Isolated enterococcal strains were propagated in MRS broth, for further isolation and purification of enterocin. A cell free solution was obtained by centrifuging (10,000rpm for 20mins, at 4^0C).The culture was adjusted to pH 7.0 by means of 1M NaOH to exclude antimicrobial effect to organic acid. This cell free culture supernatant was brought to a final ammonium sulphate concentration of 40% saturation by slow addition of salt and was stirred overnight (magnetic stirrer) at 4^0C. Then, the mixture was centrifuged (10,000 rpm for 30 mins, at 4^0C) and the surface pellicles were decanted and bottom pellets were harvested and resuspended in 10 ml of 10mM sodium phosphate buffer. After this isolation and purification the antimicrobial activity of enterocin was tested on Trypticase Soy Agar by agar well diffusion assay against S.aureus and E.coli as a target (indicator) strain. The antimicrobial activity was scored positive in presence of detectable

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clearing zone the well. The supernatant fluids of bacteria with antimicrobial activity were studied to determine the nature of inhibitor.

Results and Discussion:

Present investigation indicated that 40 strains of *Enterococci* were isolated from urine samples. The prevalence of *Enterococci* in clinical specimen can thus be attributed to their ability to grow and survive due to selective pressure of antimicrobial agents. Based on the phenotypic and biochemical identification two isolates *E.faecalis* and *E.hirae* were found in the study.

The phenotypic and biochemical identification of isolates was carried out according to the characteristics shown in Table 1.All isolates were Gram positive cocci capable of growth between 37-45^{0}C. Both *Enterococci* were found to be facultative aerobe, catalse negative and negative for haemolysin. *Enterococcus* isolates were identified on the basis of their growth on *Enterococcus* Confirmatory Agar and Bile Esculin Agar containing 40% bile. On *Enterococcus* Confirmatory Agar, the cocci produces circular, regularly shaped , low convex typical yellow colonies about 0.2mm in diameter while it shows black colonies on Bile Esculin Agar.

Sugar fermentation patterns are considered to be reliable methods of distinguishing *Enterococcus sps.*(Mundt,1986; Klein 2003) From the sugar fermentation profiles and arginine catabolism as shown in Table 1,the isolates were identified; *Enterococcus faecalis* raffinose (-), arabinose(-),mannitol (+), sorbitol (+) and arginine (+) *Enterococcus hirae* raffinose(+),arabinose(-), mannitol(+), sorbitol(-) and arginine (+).

Special tests done for identification of Enterococcus sps are tolerance to 4% and 6.5% NaCl at 45^0 c and esculin hydrolysis. Both the strains have potential to tolerate 6.5% NaCl concentration which are not acceptable to other Gram positive bacteria. In esculin hydrolysis test, blackening of bile esculin agar takes place because organism can hydrolyze esculin in presence of bile, the product esculin is formed. Esculin reacts with ferric citrate in the medium, forming phenolic iron complex which turns entire medium black.

Table 2 displays the distribution and species identification of enterococcal isolates. The majority of the isolates were E.faecalis 65% while E.hirae 35%. E.faecalis accounted for greater percentage of isolates from the samples. After screening of 68 urine samples, 40 urine samples of UTI were positive producer of enterococcus. From these, 40 Enterococcal strains against indicator strain, 26 strains (65%) exhibited antimicrobial activity. Out of 26 strains of E.faecalis 18 exhibited strong inhibition. While only 8 strains out of 14 strains of E.hirae had shown the inhibition. Inhibitory spectra of those isolates and frequency of enterocin production are represented in Table 3.The strains isolated revealed a strong inhibitory activity towards indicator strains i.e. *E.coli* and *S.aureus*.

The antimicrobial activity was thus evaluated by measuring the diameter of zone of inhibition. Enterocin produced were analyzed to check its efficiency to inhibit the indicator strains. It was found that both strains *E.coli* and *S.aureus* were strongly inhibited by enterocin confirming that enterocin shows inhibitory activity against indicator strains. Thus, it is concluded that isolation of Enterococcus species is beneficial as it produces a protein which have bactericidal property known as enterocin and can prevent growth of various Gram positive and Gram negative bacteria demonstrating its potential as novel therapeutic agent.

Table 1. Morphological and Biochemical Characterization of Enterococcus isolates.

Characteristics	Isolates	
	E.faecalis	E.hirae
Cell Morphology	Cocci	Cocci
Gram reaction	Gram positive	Gram positive
Colony Characters		
Size	0.2mm	0.1-0.2mm
Colony shape	Circular	Circular
Elevation	Convex	Convex
Opacity	Opaque	Opaque
Colour on Enterococcus Confirmatory Agar	Yellow colony	Yellow colony
Bile Esculin Agar	Black colony	Black colony
Catalse	Negative	Negative
Haemolysin production	Negative	Negative
CO_2 production from glucose	Positive	Positive
Hydolysis of Esculin	Positive	Positive
Growth in presence of NaCl		
4%	positive	positive
6.5%	positive	positive
Utilization of Carbohydrate		
L-arabinose	Negative	Negative
Ribose	positive	positive
Sucrose	positive	positive
Mannitol	positive	positive
D-raffinose	Negaitive	positive
Lactose	positive	positive
Sorbitol	positive	Negative

Table 2.Frequency Distribution of Enterococcus isolates

Identified strains	No.of strains
E.faecalis	26
E.hirae	14
Total	40

Table 3. Frequency of enterocin production screened by Agar Well Diffusion Method

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

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