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IDENTIFICATION OF ENTEROCIN FROM ENTEROCOCCUS CASSELIFLAVUS ISOLATED FROM SOYBEAN

Rajarajan P.N.[1]*, Rajeswari K.[2], Jeganathan P.[3], and Sumathy N.[4]

[1,2,3,4]Assistant Professor, Dept. of Microbiology, The Madura College (Autonomous), Madurai, Tamilnadu, India.

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***Corresponding Author**
Rajarajan P.N.
Assistant Professor, Dept. of Microbiology, The Madura College (Autonomous), Madurai, Tamilnadu, India.

ABSTRACT

Background: In the present study, *Enterococcus casseliflavus* was isolated from soybean and enterocin was produced. Partial purification of enterocin, its antibacterial activity, sensitivity to pH, temperature, organic solvents, metal ions, molecular weight determination and protein estimation were investigated. The objective of the present study is to isolate and identify Enterococcus species from soybean, isolation of food borne pathogens which act as indicator organism like *Staphylococcus* from raw milk, *Bacillus subtilis* from soil, *Escherchia coli* and *Vibrio parahaemolyticus* and *Enterococcus faecalis* from sewage water, Production and purification of bacteriocins, Determination of bactericidal activity of enterocins produced by enterococcus against food borne pathogens, Molecular weight determination of enterocin by SDS-PAGE, Estimation of protein by Lowry's method and physical and biochemical characterization. **Methods:** The cell free neutralized supernatant was obtained by removing the cells by centrifugation and neutralizing it by adjusting the pH to 7. The cell free supernatant exhibited antibacterial activity against the food pathogens which were isolated from various sources. It inhibited the growth of *Bacillus subtilis, Escherchia coli, Enterococcus faecalis* and *Vibrio parahaemolyticus* but not *Staphylococcus aureus.* Enterocins were purified from the culture supernatant by ammonium sulphate precipitation and dialysis. **Results:** The molecular weight of the enterocin was determined by SDS-PAGE and was found to be 4000Da approximately. The amount of protein present in crude enterocin was estimated by Lowry's method and was found to be 336µg/ml. The sensitivity of bacteriocin to various pH, temperature, organic solvents and metal ions was determined. The bacteriocin was stable at a pH range of 4-9 and at a temperature of 80^0C, 100^oC and 121^0C.

Conclusion: The possible use of bacteriocins as food biopreservatives could lead to the replacement of synthetic chemical preservatives, which have their antimicrobial action reduced due the continued appearance of multiresistant microbial lineages.The isolated active compounds from *Enterococcus casseliflavus* was proteinaceous, heat stable. It was also stable and active over a wide pH range and had a bacteriocidal mode of action on various organisms. This enterocin was a low molecular weight protein and heat stable. So we can derive these under class II bacteriocins.

KEY WORDS: Bacteriocins, Antimicrobials, Food Industry, Biopreservation.

INTRODUCTION

Microbes produce a huge abundance and diversity of microbial defense systems including antibiotics, exotoxins, lytic agents, metabolic biproducts and bacteriocins[1]. Bacteriocins are the most varied and plentiful group of all the microbial defense system and may be characterized as "the microbial weapon of choice".[3]

Bacteriocins are ribosomally synthesized bacterial peptides or proteins which show antimicrobial activity generally against closely related species to producers[4]. They were originally defined as bactericidal proteins characterized by lethal biosynthesis, a very narrow range of activity and adsorption to specific cell envelope receptor.[2]

Pasteur and Joubert reported the occurrence of antagonistic interactions among bacteria. These researchers observed that a bacterial isolate was able to interfere on the *Bacillus anthracis* growth[1]. At the same way, inhibitory action exerted by a *Staphylococcus* spp. isolate when in interaction with *Corynebacterium diptheriae* was also observed. This discovery led, at that time, the application of *Staphylococcal* isolates as useful procedure to be applied in diphtheria treatment and this therapeutic use was made by steamer form. Gátia first elucidated the inhibition mechanisms occurring in these processes of microbial interactions. It was observed that *E. coli* cells produced in liquid medium a substance stable to temperature oscillations and also possessors of inhibitory activity on the growth of other taxonomically similar microorganisms. It was, probably, the initial step for studies involving bacteriocins[1]. Originally, these substances were denominated colicins, because most of the studies observed their synthesis by *E. coli* cells. After verifying that the synthesis of these molecules was common among other bacteria, it was chosen the name bacteriocin to

designate them.[2] Bacteriocins have been detected in all major lineages of Eubacteria and Archaebacteria.[5]

Bacteriocins are antimicrobial proteins or peptides that are usually inhibitory only to strains closely related to the producing bacteria. Many bacteriocins from Gram- positive bacteria have fairly broad inhibitory spectra, and these bacteriocins may therefore have an applied potential as antimicrobial agents. In particular, bacteriocins from lactic acid bacteria have become attractive as natural food preservatives. Lactic acid bacteria have been used in food and feed preservation for centuries, and their preservative effects are mainly due to the formation of large amounts of lactic acid. This is not always sufficient to prevent bacterial spoilage or outgrowth of pathogenic bacteria, but bacteriocin producing lactic acid bacteria may serve as a replacement for chemical preservatives in foods.[6] The broad spectrum bacteriocins from lactic acid bacteria that have been characterized are all small (2.5-6 kDa), heat stable, hydrophobic and cationic.

The present study is to determine the antibacterial activity of enterocins produced by *Enterococcus casseliflavus* against food borne pathogens and determine its molecular weight.

MATERIALS AND METHODS

Sample collection

Soybean: fresh soybeans were collected from the market.

Soil: The soil sample was collected aseptically in a sterile polythene bags using sterile sampling devices and taken to the laboratory for further processing.

Sewage water: sewage water was collected aseptically in a sterile container and taken to the laboratory for further processing.

Raw milk: The milk sample was collected aseptically in a sterile screw capped container.

Isolation of *Enterococcus* from soybean

Soybean were dipped in sterile distilled water and crushed. The crushed sample was streaked on enterococcus isolation agar. The plates were incubate at 37^0C for 24 hours. Colonies from the above plates were gram stained and various tests were performed to identify the species.

Isolation of food borne pathogens

Isolation of *Bacillus subtilis* from soil

1g of soil was weighed and mixed with 10ml of sterile distilled water. This was serially diluted and 0.1ml of the sample was spreaded on nutrient agar and incubated at 37^0C for 24 hours. Suspected colonies were gram stained and further tests were performed for confirmation.

Isolation of *Escherchia coli*, *Enterococcus facealis* and *Vibrio parahaemolyticus* from sewage

1ml of sewage sample was mixed with 99ml of sterile distilled water. This was streaked on EMB, Slanetz and Bartley agar and TCBS plate and incubated at 37^0C for 24 hours. Suspected colonies were gram stained and further tests were done for confirmation.

Isolation of S*taphylococcus aureus* from raw milk

1ml of raw milk sample was inoculated in Thioglycollate broth and incubated at 37^0C for 24 hours. After incubation it was streaked on MSA plates and kept for incubation at 37^0C for 24 hours. Suspected colonies from MSA plates were gram stained and confirmed by further tests.

Extraction of Enterocin

The isolated organism was inoculated in 10ml of lactobacillus MRS broth ($_P$H 7.0) and incubated at 37^0C for 24 hours. The bacterial cells were separated by centrifugation at 10,000g for 30 minutes at 4^0C. The cell free supernatant was obtained and its $_P$H was adjusted to 7 with 1m NaOH. The supernatant was added with 30,40,50,60 % (W/V) ammonium sulphate and stirred for 2 hours at 4^0C. The precipitate obtained was resuspended in 50mM sodium phosphate buffer (Ph 7). Then dialysis was carried out with same buffer for 24 hours at 25^0C. The dialysate formed was collected by centrifugation. The pellet was resuspended in sodium phosphate buffer and used for further studies.

Determination Enterocin activity

Enterocin activity can be determined by agar well diffusion method. The food borne pathogens isolated from various sources were inoculated in nutrient broth and incubated at 37^0C for 16-18 hours. 10ml of nutrient agar medium with 1.5% agar was prepared as bottom layer. Later 5ml of soft agar (0.75%) was seeded with 10µl of the overnight culture and overlaid on the hard agar. The wells were punctured in the plates and filled with 20µl of

enterocin. The plates were incubated at 37^0C for 24 hours and examined for inhibitory zones around the wells.

Determination of molecular weight of enterocins

The molecular weight of enterocins was determined by SDS-PAGE. The glass plates were sealed and they are checked for leakage. 5ml of separating gel was poured in between the plates without any air bubble and it was overlaid by n-butanol. Later it was allowed to polymerise. Later after polymerization, the n- butanol was poured off and the comb was inserted. The stacking gel was poured to it without any air bubble, it was allowed to polymerise. Later after polymerization, the comb was removed carefully and the wells were rinsed with tank buffer. The bottom seal of the plates was removed and the plate was placed in the tank. 0.1 ml of the extract was dissolved in 0.1ml of the sample buffer and from this 20µl was loaded in the well along with molecular weight leader marker in the I well in which III well posses the sample. After electrophoresis process, the gel was stained with Coomassie brilliant blue for 3 hours and destained. The molecular weight of the enterocins was determined by calculating the Rf values and plotting a semi log graph with molecular weight on X- axis and Rf on Y-axis.

Estimation of protein

The amount of protein present in the sample was estimated by Lowry's method. 0.2, 0.4, 0.6……1ml protein solution was pippeted out and the total volume was made up to 4ml with distilled water. Water alone serves a s blank 5.5ml of the alkaline mix was added, mixed well and allowed to stand at room temperature for 10-15 minutes. 0.5ml of Folin-ciocalteau reagent was added to each tube and mixed. Tubes were left for 30 minutes till color appeared. Blue color was measured at 650nm and standard graph was plotted.

Physical and biochemical characterization

Effect of $_P$H

Enterocin preparation was adjusted to different pH level between 3.0 to 10.0 with 1M NaOH or 1M HCL. Samples were maintained for 1 hour at 29^0C. They were then assayed for bioactivity by agar well diffusion method.

Effect of temperature

Thermpostability of enterocin preparation was determined by heating 2ml of the preparation at 60^0C, 80^0C and 100^0C for 15 minutes. Samples were removed, cooled and assayed for residual activity.

Effect of organic solvents

Equal volume of crude enterocin was mixed with 1% organic solvents. Samples were stirred and incubated at 29^0C for 2 hours and further processed through agar well diffusion assay.

Effect of Metal ion

Equal volume of crude enterocin was mixed with 1Mm solution of different metal ions. Samples were stirred and incubated at 29^0C for 2 hours and further processed through agar well diffusion assay.

RESULTS

Identification of Enterococcus species

Fresh soybeans were used for the isolation of Enterococcus species. Two different types of colonies were observed on Enterococcus isolation agar.

The colonies were streaked individually on trypticase soy agar and observed for pigmentation. Only one colony was found to produce a non diffusible yellow pigment. Such colony is selected identified and screened for bacteriocin production.

MORPHOLOGY

Gram positive cocci in single, pairs and small chains. Motile. The species was confirmed by further test were tabulated (Table 1).

Table 1: Biochemical tests for *Enterococcus casseliflavus.*

Test	**Result**
Catalase	Negative
Oxidase	Negative
Growth in 6.5%NaCl	Positive
Esculin hydrolysis	Positive
Sugar Fermentation Test	
Arabinose	Positive
Raffinose	Negative
Adaulcitol	Negative
Mannitol	Positive
Sorbitol	Negative

Identification of food borne pathogens

I. *Bacillus subtilis*

Nutrient agar

Colonies are white, granular, dry with irregular edges, wavy.

Morphology

Gram positive bacilli in chain, motile and sporulated. The species was identified by performing biochemical tests and results were tabulated (Table 2).

Table 2: Biochemical tests for *Bacillus subtilis.*

Test	**Result**
Catalase	Positive
Oxidase	Negative
Indole	Negative
Methyl Red	Negative
Voges proskauer	Positive
Citrate utilization test	Negative
Sugar Fermentation Test	
Glucose	Positive
Sucrose	Positive
Mannitol	Positive
Xylose	Positive
Arabinose	Negative

II. *Escherichia coli*

Eosin methylene blue agar

Colonies are smooth and produced greenish metallic sheen.

Morphology

Gram negative rods, actively motile. The organism was further confirmed by biochemical tests and tabulated (Table 3).

Table 3: Biochemical tests for *Escherichia coli.*

Test	**Result**
Catalase	Positive
Oxidase	Negative
Indole	Positive
Methyl Red	Positive
Voges proskauer	Negative
Citrate utilization test	Negative
Urease	Negative
TSI	A/A, H_2S^-,G^+

III. *Staphylooccus aureus*

Mannitol Salt Agar

The strain produced golden yellow pigment and colonies are pin pointed.

Morphology

Gram positive cocci in clusters. Further test were done to confirm the organism and results were tabulated (Table 4).

Table 4: Biochemical tests for *Staphylooccus aureus.*

Test	Result
Catalase	Positive
DNase	Positive
Coagulase	Positive

IV. *Vibrio parahaemolyticus*

Thiosulphate citrate bile salt sucrose agar

The organism produced small smooth convex green color colonies.

Morphology

Gram negative bacilli, motile. The species was further confirmed by biochemical tests and tabulated (Table 5).

Table 5: Biochemical tests for *Vibrio parahaemolyticus.*

Test	Result
Catalase	Positive
Oxidase	Positive
Indole	Positive
Methyl Red	Negative
Voges proskauer	Negative
Citrate utilization test	Positive
Urease	Negative
Carbohydrate fermentation	
Sucrose	Positive
Arabinose	Negative

V. *Enterococcus faecalis*

Slanetz and Bartley agar

Colonies are red, smooth, regular and pin pointed.

Morphology

Gram positive cocci in pairs and non motile. The species was further confirmed by biochemical tests and tabulated (Table 6).

Table 6: Biochemical tests for *Enterococcus faecalis.*

Test	**Result**
Catalase	Negative
Oxidase	Negative
Growth in 6.5% Nacl	Positive
Esculin hydrolysis	Positive
Carbohydrate fermentation test	
Arabinose	Negative
Raffinose	Negative
Dulcitol	Negative
Mannitol	Positive
Sorbitol	Positive

Determination of Enterocin activity

Zone of inhibition was observed for *Enterococcus faecalis*, *Bacillus subtilis*, *Escherichia coli* and *Vibrio parahaemolyticus* but not for staphylococcus aureus. The diameter of the zone was measured and results were tabulated (Table 7).

Table 7: Determination of Enterocin activity.

organism	**Zone Of Inhibition (mm)**
Bacillus subtilits	10
Enterococcus faecalis	8
Escherichia coli	5
Staphylococcus aureus	-
Vibrio parahaemolyticus	5

Determination of molecular weight of Enterocin

The molecular weigt of enterocin was determined by SDS-PAGE. The relative factor (Rf) of the bands was obtained. The Rf value of the bands obtained along with the molecular weight of the marker are given in the table (Table 8).

The molecular weight of enterocin was determined by plotting a semi-log graph with Rf values on X-axis and molecular weight of the marker on Y-axis.

Table 8: Determination of molecular weight of Enterocin.

Molecular weight (Daltons)	Distance travelled by dye (cm)	Distance travelled by band (cm)	Rf value
Marker: (Lane I)			
43000	4.5	1.2	0.26
29000	4.5	1.7	0.37
20100	4.5	2.2	0.48
14300	4.5	2.9	0.64
6500	4.5	3.5	0.77
3000	4.5	4.0	0.88
Sample (Lane 3)	4.5	3.8	0.84

Estimation of Protein

Optical density was measured and results were tabulated (Table 9). The graph was plotted taking concentration of protein (µg/ml) on X-axis and O.D values in Y-axis and the amount of protein was estimated.

Physical and biochemical characterization

The effect of $_{P}$H, temperature, organic solvents and metal ions were studied and results were tabulated (Table 10, 11, 12, 13).

Table 9: Estimation of protein by Lowry's method.

Protein Stock	O.D
0.2	0.34
0.4	0.42
0.6	0.50
0.8	0.61
1.0	0.66
T_1	0.35

Table 10: Effect of $_{P}$H on Enterocin activity.

$_{P}$H	Zone Of Inhibition
3	-
4	+
5	+
6	+
7	+
8	+
9	+
10	-

Table 11: Effect of temperature on Enterocin activity.

Temperature	Zone Of Inhibition
80^0C/15 MIN	+
100^0C/15 MIN	+
121^0C/15 MIN	+

Table 12: Effect of organic solvents on Enterocin activity.

Organic Solvents	Zone Of Inhibition
Ethanol	+
Methanol	+
Butanol	+
Chloroform	+

Table 13: Effect of metal ions on Enterocin activity.

Metal Ions	Zone Of Inhibition
$MgSO_4$	+
$CaCl_2$	+
$(NH_3)_2$ NO_3	+
$MnSO_4$	+

DISCUSSION

Lactic acid bacteria and their bacteriocins have been the focus of many research programs due to industrial importance of lactic acid bacteria and their contribution of the well being of humans. Bacteriocins differ from classical antibiotics. As such, antibiotics are synthesized by unique enzymatic systems, in contrast to bacteriocins, which are ribosomally synthesized and which have a much more narrow target specificity than antibiotics. Each bacteriocin has its own dedicated immunity protein whose gene is linked to the bacteriocin gene, whereas genetic determinants for antibiotic resistance are not linked and are expressed independently of the genes encoding the antibiotic synthesis apparatus. Bacteriocins are usually produced in the growth phase and production is frequently regulated by a two component regulatory system, while antibiotics are secondary metabolites produced in the stationary phase.[7]

In this study, identification, characterization and production of bacteriocin by *Enterococcus casseliflavus* isolated from soybean was described. Although various bacteriocins producing lactic acid bacteria strains including enterococci were isolated so far from plant-related origins, a few examples have been reported from soybean- related origins such as fermented soybean: Malaysian Tempeh[8] and Japanese Miso.

The isolated bacteriocin producing lactic acid bacteria strain from raw soybean. Thus bacteriocin producing strains could be isolated from both raw materials and their fermented foods. This fact implies that bacteriocin producers found in fermented foods could originate from raw materials.[9]

Enterococcus casseliflavus was mostly isolated from plant related origins whereas *Enterococcus faecium* and *Enterococcus faecalis* are known to be isolated from a variety of sources especially from animal related sources such as dairy and meat products and intestinal tracts[10]. Most of the enterococcal bacteriocins so far discovered from isolates belonging to *Enterococcus faecium* and *Enterococcus faecalis* and they are expected to be applied for manufacturing meat and dairy products.[11] However bacteriocins from plant associated enterococcal strains might be more suitable for preservation of plant related foods.

The Bacteriocin producd by *Enterococcus casseliflavus* was highly stable at wide range of pH and against heat treatment, organic solvents and metal ions. Enterocin was active against *Enterococcus faecalis, Bacillus subtilis, Escherchia coli* and *Vibrio parahaemolyticus* but not *Staphylococcus aureus.*

Bacteriocin from *Lactobacillus acidophilus* was purified by ammonium SO4 precipitation. The same method was followed in this study to purify enterocin. Molecular size of enterocin was determined using SDS-PAGE gel. The molecular weight of enterocin was found to be 4000Da which was approximately equal to molecular weight of enterocin from *Enterococcus casseliflavus.*[12]

The estimated protein of pediocin PA-1 as 20µg/ml, leuconocin S as 48.5µg/ml and Lactacin F as 13.5µg/ml. In the same manner protein concentration was estimated as 336µg/ml for enterocin in study. Further work will be necessary to extend the knowledge of this enterocin and to determine whether the genes encoding enterocin production is located on the chromosome or plasmid DNA and to screen its probiotic properties.

SUMMARY AND CONCLUSION

In the present study, *Enterococcus casseliflavus* was isolated from soybean and enterocin was produced. Partial purification of enterocin, its antibacterial activity, sensitivity to pH, temperature, organic solvents, metal ions, molecular weight determination and protein estimation were investigated.

The cell free neutralized supernatant was obtained by removing the cells by centrifugation and neutralizing it by adjusting the pH to 7. The cell free supernatant exhibited antibacterial activity against the food pathogens which were isolated from various sources. It inhibited the growth of *Bacillus subtilis*, *Escherchia coli*, *Enterococcus faecalis* and *Vibrio parahaemolyticus* but not *Staphylococcus aureus.* Enterocins were purified from the culture supernatant by ammonium sulphate precipitation and dialysis.

The molecular weight of the enterocin was determined by SDS-PAGE and was found to be 4000Da approximately. The amount of protein present in crude enterocin was estimated by Lowrys method and was found to be 336µg/ml. The sensitivity of bacteriocin to various pH, temperature, organic solvents and metal ions was deteromined. The bacteriocin was stable at a pH range of 4-9 and at a temperature of 80^0C, 100^oC and 121^0C.

In recent years the frequency of antibiotic resistant bacteria causing human and animal diseases has increased dramatically. These antibiotic resistant bacteria are found in our environment including food and they represent a serious health problem. There is increasing concern about the use of synthetic chemicals as food preservatives due to their toxicity. Consequently there is demand of new antibiotics and preservatives. Thus, they might be developed as an alternative or a supplement to traditional antibiotics and chemical preservatives.

In conclusion the bacetriocin producing culture can be used as starter culture for the food fermentation milk products like cheese, yoghurt etc to reduce food spoiling bacteria.

The isolated active compounds from *Enterococcus casseliflavus* was proteinaceous, heat stable. It was also stable and active over a wide pH range and had a bacteriocidal mode of action on various organisms. This enterocin was a low molecular weight protein and heat stable. So we can derive these under class II bacteriocins.

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