



RESEARCH ARTICLE

ANTIBACTERIAL AND BIOPRESERVATIVE EFFECT OF BACTERIOCIN PRODUCED BY *ENTEROCOCCUS FAECIUM*.

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Abstract

Bacteriocin producing lactic acid bacteria (LAB) from fermented foods are of great significance because of their potential use as antibacterial activity against similar or closely related bacterial strains. The present study was focused on detection of antibacterial activity of bacteriocin producing LAB from fermented green gram batter. The three isolates (A3R, A5R and A8R) were identified as *Enterococcus faecium* by 16S ribosomal DNA (rDNA). The bacteriocin of three isolates was evaluated for antagonistic activity against indicator organisms, *Pseudomonas aeruginosa*, *Staph aureus* and *Escherichia coli*. The bacteriocin of A3R and A5R inhibited the growth of *P. aeruginosa* and *S. aureus*. The growth of *E. coli* was inhibited by bacteriocin of A5R and A8R, not inhibited by bacteriocin of A3R. The study revealed high bacteriocin production by A5R. The sensitivity of the extracted bacteriocin to proteolytic enzymes, indicated the proteinaceous nature of bacteriocin. The preservative effect of ascorbic acid was enhanced in presence of bacteriocin of A3R, indicating its suitability as biopreservative agent in coconut water. The isolation and identification of *Enterococcus faecium* is one of the lactic acid bacteria involved in green gram fermentation and production of bacteriocin.

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Introduction:-

Lactic acid bacteria (LAB) is an important group of industrial microorganism involved in the processing of various fermented food which include vegetables and sausages, dietary adjuncts, probiotics and even cosmetic ingredients. LAB is used as starter culture to improve the texture and the flavour of the products. The ability to inhibit growth of spoilage microorganisms and pathogenic bacteria contribute to the maintenance of hygienic and quality of the products or host health. This inhibitory activity is the result of the metabolic product secreted by these LAB which acts as antimicrobial compounds. These compounds include organic acids, diacetyl, hydrogen peroxide and bacteriocin (Barefoot and Klaenhammer, 1983).

Bacteriocins, are defined as bioactive peptides or protein, extracellularly released, with an antimicrobial activity towards gram positive bacteria including closely related species and/or food spoilage and pathogenic bacteria such as *Bacillus cereus*, *Clostridium botulinum*, *Staphylococcus aureus* and *Listeria monocytogenes* (Nettles and Barefoot, 1993). Although the bacteriocins are produced by many Gram-positive and Gram-negative bacteria, those produced by LAB are of particular interest because known as "good bacteria" used as natural preservatives in food

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industry (Paul et al., 2002). During the last few years a large number of new LAB bacteriocins have been identified and characterized. However only few have been described to possess activities against Gram-negative bacteria, e.g. plantaricin 35d produced by *Lactobacillus plantarum* (Meesi et al., 2001), Bacteriocin ST34BR produced by *L.lactis subsp. Lactis* (Todorov et al., 2004c).

The use of bacteriocin or bacteriocin producing culture as potential 'biopreservatives', and possibly for replacing chemical preservatives (Abee et al., 1995) has received much attention. This is due to current awareness of consumers towards the use of food preservatives. Therefore the purpose of this study was aimed at isolation of lactic acid bacteria from green gram (*Vigna radiata*) fermented batter, for their ability to produce bacteriocin which exhibit antagonistic activity and biopreservative property.

Material and Methods:-

Sample collection and preparation:-

Green gram was collected, cleaned, soaked in water for 8 hr and batter was prepared. It was allowed to ferment at room temperature overnight (O/N).

Isolation and identification of bacteriocin producing Lactic acid bacteria (LAB):-

The fermented batter of 1 ml was added to 9 ml of distilled water, serial dilutions were performed up to 10^{-7} and plated on Man, Rogosa and Sharpe (MRS) agar. The plates were incubated at 37°C for 24 hr. After incubation, pure culture was made on MRS agar and tested for bacteriocin production (Nettles and Barefoot, 1993). Then the isolates were Gram stained and examined microscopically. Based on Bergey's Manual of Systemic Bacteriology (Ravi et al., 2011), isolates were tested for their ability to ferment dextrose, lactose, sucrose and mannitol. Catalase activity was tested by spotting colonies with 3% hydrogen peroxide and Oxidase by using oxidase disc.

Carbohydrate fermentation test:-

The carbohydrate fermentation of the LAB isolates using the dextrose (1%), lactose (1%) sucrose (1%) and mannitol (1%) was identified by using basal medium (peptone water). The inverted Durham's tubes were placed in the medium to detect the formation of gas. Incubation was carried out at 37°C for 18-48 hr and results were recorded at 18-24 hr. The methylene blue was used as an indicator, positive result (acid production) was indicated by yellow colour and negative result (no acid production) was indicated by blue colour (Kozaki et al., 1992).

Antibiotic susceptibility test:-

Antimicrobial susceptibility testing of isolates were performed using commercially available predefined gradient of antibiotic concentrations on a plastic strip (Hi-Media) by using nutrient agar.

Production and concentration of bacteriocin in culture supernatant:-

Isolates were grown in MRS broth at 37°C for 48 hr under rotatory (incubate shaker) condition. After incubation, the broth was centrifuged at 5000 rpm for 10 min and the cells were separated (Esayas et al., 2008). The cell free supernatant was used as crude bacteriocin. The bacteriocin reacted with Folin Ciocalteau reagent, Lowry method (Lowry et al., 1951) and formed a complex which is blue purple color complex which was read at 660 nm in spectrophotometer. Bovine serum albumin (BSA) was used as a standard protein.

Antibacterial activity of bacteriocin against indicator organisms:-

The antibacterial activity of bacteriocin of all three isolates was tested against indicator organisms, Gram positive organism (*Staphylococcus aureus*) and Gram negative organisms (*Escherichia coli*, *Pseudomonas aeruginosa*,). The pH of culture supernatants was adjusted to pH 5 and pH 7 using 1M NaOH. The antimicrobial activity of bacteriocin against pathogenic microorganisms was determined by agar well diffusion method (Gomez et al., 2002). Nutrient agar plates were inoculated with 100 ul (Nutrient broth containing O/N culture) of each indicator microorganism by spread plate method and inhibitory activity of bacteriocins against indicator organisms was tested by agar well diffusion method.

Antibacterial activity of bacteriocin in presence of proteinase K:-

The proteinase K (1mg/ml) was added to culture supernatant (bacteriocin), incubated at 37°C for 1 hr and evaluated for antibacterial activity of bacteriocin of three isolates by agar well diffusion (Diaz et al., 1993).

Preservative property of ascorbic acid in presence of A3R bacteriocin in coconut water:-

Coconut was obtained aseptically in the laboratory. The preservative effect of ascorbic acid as chemical preservative (CP) in presence of bacteriocin as biopreservative (BP) was investigated. The set of seven boiling tubes were used for the experiment. To all seven tubes 5 ml of coconut water was added. First tube was the control (no preservative was added). To the second tube BP (1%), third CP (1%), fourth BP (4%), fifth CP (4%) were added. The sixth and seventh tubes contained combination of both BP and CP. To the sixth tube (BP 1% + CP 1%) and seventh tube (BP 4% + CP 4%) were added. All the seven tubes were incubated at room temperature for 24 hr, 100 ul of sample from each flask was inoculated on nutrient agar medium by spread plate method, incubated at 37°C for 24 hr and observed for microbial colonies.

Sequence of Isolates A3R, A5R and A8R:-

The sequencing of three isolates A5R and A3R were analysed using the method, 16S rDNA, done by Zeal Biologicals, Secunderabad, Telangana State.

Results:-**Isolation and identification of bacteriocin producing Lactic acid bacteria (LAB):-**

The three isolates A3R, A5R and A8R, bacteriocin producing Lactic acid bacteria were isolated from fermented green gram batter, identified as *Enterococcus faecium* based on their physiological, biochemical (Table 1) and molecular characteristics by 16S rDNA. The isolates A3R, A5R and A8R were Gram-positive cocci, catalase negative, acid production in dextrose and lactose fermentation and no hemolysis, having small, smooth round colonies on MRS agar medium.

Table 1:-General Properties of Isolates

SAMPLE	ISOLATE	GENERAL PROPERTIES	IDENTIFIED ORGANISM
Green gram	A3R	Gram positive cocci, Catalase -ve, Oxidase-ve, Coagulase-ve, no hemolysis, acid production in dextrose and lactose	<i>Enterococcus faecium</i>
Green gram	A5R	Gram positive cocci, Catalase -ve, Oxidase-ve, Coagulase-ve, no hemolysis, acid production in dextrose and lactose	<i>Enterococcus faecium</i>
Green gram	A8R	Gram positive cocci, Catalase -ve, Oxidase-ve, Coagulase-ve, no hemolysis, acid production in dextrose and lactose	<i>Enterococcus faecium</i>

-ve: Negative

Carbohydrate fermentation test:-

The carbohydrate fermentation pattern (Table 2) of three isolates (A3R, A5R and A8R) using the dextrose (1%), lactose (1%) sucrose (1%) and mannitol (1%) in peptone water.

Table 2:- Carbohydrates fermentation of isolates

ISOLATE	DEXTROSE		LACTOSE		SUCROSE		MANNITOL	
	AP	GP	AP	GP	AP	GP	AP	GP
A3R	+	-	-	-	+	-	-	-
A5R	+	-	-	-	+	-	-	-
A8R	+	-	-	-	+	-	-	-

AP: Acid production, GP: Gas production,

AP +: Presence of Acid production, AP -: No Acid production, GP -: No gas production

Antibiotic susceptibility test:-

The antibiotic susceptibility testing of isolates indicated in Table 3. The three isolates were resistant to all twelve antibiotics.

Table 3:- Antibiotic susceptibility testing of isolates

ANTIBIOTIC	A3R	A5R	A8R
Amikacin	r	r	r
Ampiclox	r	r	r
Ciprofloxacin	r	r	r
Clarithromycin	r	r	r
Cefotaxime	r	r	r
Sparfloxacin	r	r	r
Cefuroxime	r	r	r
Cefoperazone	r	r	r
Gentamicin	r	r	r
Roxithromycin	r	r	r
Cefadroxil	r	r	r
Azithromycin	r	r	r

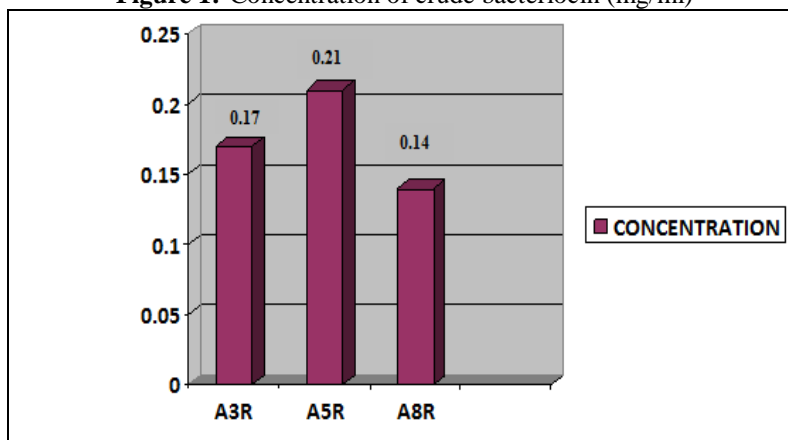
r: Resistant

Production and concentration of bacteriocin in culture supernatant:-

The bacteriocin production (Table 4 and Figure 1) was estimated in culture supernatant of isolates A3R, A5R and A8R at 37°C under rotatory condition (Incubate shaker). The concentration of bacteriocin was 0.17 mg/ml (A3R), 0.21 mg/ml (A5R) and 0.14 mg/ml (A8R). The highest production of bacteriocin was observed with A5R.

Table 4:-Concentration of bacteriocin in culture supernatants of A3R, A5R and A8R

ISOLATE	CONCENTRATION OF PROTEIN mg/ml
A3R	0.17
A5R	0.21
A8R	0.14

Figure 1:-Concentration of crude bacteriocin (mg/ml)

A3R:- Bacteriocin production at 37°C under rotatory condition (Incubate shaker)

A5R:- Bacteriocin production at 37°C under rotatory condition (Incubate shaker).

A8R:- Bacteriocin production at 37°C under rotatory condition (Incubate shaker).

Antibacterial activity of bacteriocin against indicator organisms:-


Tables 5, 6, 7, 8 indicate the antibacterial activity of three isolates at pH 5 and pH 7. The bacteriocin of A5R and A8R inhibited the growth of *E. coli* and *P. aeruginosa* and *S. aureus* at pH5. The bacteriocin of A35R inhibited the growth of *P. aeruginosa* and *S. aureus* at pH5. The indicator organisms were resistant to bacteriocin of three isolates at pH7.

Table 5:-Antibacterial activity of bacteriocin

ISOLATES	ZONE OF INHIBITION (mm)					
	<i>E.coli</i>		<i>P.aeruginosa</i>		<i>S.aureus</i>	
	pH5	pH7	pH5	pH7	pH5	pH7
A3R	-	-	16	-	14	-
A5R	12	-	18	-	17	-
A8R	12	-	15	-	16	-


(-): No zone of inhibition

Table 6:-Antibacterial activity of bacteriocin of three isolates with *E.coli*

ISOLATES	ZONE OF INHIBITION (mm) pH 5 <i>E.coli</i>
A3R	r
A5R	s
A8R	s
Picture	


r: resistant (No zone of inhibition) s: sensitive (Presence of zone of inhibition)

Table 7:-Antibacterial activity of bacteriocin of three isolates with *P.aeruginosa*

ISOLATES	ZONE OF INHIBITION (mm) pH 5 <i>P.aeruginosa</i>
A3R	s
A5R	s
A8R	s
Picture	

s: sensitive (Presence of zone of inhibition)

Table 8:-Antibacterial activity of bacteriocin of three isolates with *S.aureus*




ISOLATES	ZONE OF INHIBITION (mm) pH 5 <i>S.aureus</i>
A3R	s
A5R	s
A8R	s
Picture	

s: sensitive (Presence of zone of inhibition)

Antibacterial activity of bacteriocin in presence of proteinase K:-

The sensitivity of bacteriocin of isolates (A3R, A5R and A8R) to proteinase K was evaluated (Table 9), indicator organisms were resistant to bacteriocin, indicating loss of antibacterial activity.

Table 9:-Antibacterial activity of bacteriocin in presence of Proteinase K








ISOLATES	ZONE OF INHIBITION (mm)		
	<i>E.coil</i>	<i>P.aeruginosa</i>	<i>S.aureus</i>
A3R	r	r	r
A5R	r	r	r
A8R	r	r	r
Pictures			

r: resistant (No zone of inhibition)

Effect of preservative property of ascorbic acid in presence of A3R bacteriocin in coconut water:-

The bacteriocin of A3R as biopreservative (BP) agent in coconut water (Table 10) was investigated based on the number of colonies. The number of colonies reduced in the presence of bacteriocin (1% & 4%) and ascorbic acid (1% & 4%) as chemical preservative (CP). The maximum reduction in the number of colonies was observed in the combination of BP + CP (1% + 1%). The preservative effect was further enhanced in the combination of increased concentration of BP + CP (4% + 4%), indicated by presence of few colonies.

Table 10:- Preservative property of ascorbic acid in presence of A3R bacteriocin in coconut water

COCONUT WATER + PRESERVATIVE	MICROFLORA (COLONIES)	PICTURES
Only coconut water (Control)	Lawn of growth	
Coconut water + BP (1%)	Decrease in the number of colonies	
Coconut water + CP (1%)	Decrease in the number of colonies	
Coconut water + BP (4%)	Maximum reduction in the number of colonies	
Coconut water + CP (4%)	Maximum reduction in the number of colonies	
Coconut water + B P(1%) + CP (1%)	Decrease in the number of colonies	
Coconut water + BP (4%) + CP (4%)	Very few colonies were observed	

BP:-Bacteriocin as Biopreservative (Concentration:1% & 4%)

CP:-Ascorbic acid as Chemical preservative (Concentration:1% & 4%)

Sequencing results of Isolates A3R, A5R and A8R:-

The sequencing of two isolates A5R and A3R were analysed by 16S rDNA and identified as:

1. A3R - *Enterococcus faecium* (NRIC 0114; AB362603.E).
2. A5R - *Enterococcus faecium* (NRIC 0114; AB362603.E).
3. A8R - *Enterococcus faecium* (NRIC 0114; AB362603.E).

Discussion:-

The LAB is considered as “food grade” organisms, show special promise for selection and implementation as protective cultures. In addition, some LAB exhibit potent antimicrobial activities in the form of small, heat-stable, antimicrobial peptides called bacteriocins (Riley and Wertz, 2002). The bacteriocins are proteinaceous compounds that are able to inhibit a wide variety of organisms, mostly closely related to the producer organisms (Mandal et al., 2011).

The three isolates showed the activity of acidification with dextrose and sucrose and produced no gas. The two isolates didn't ferment lactose and mannitol. *Lact. plantarum* 20B produced lactic acid when maltose was used, and markedly increased growth rate and the production of acetic acid (Kandler, 1983).

The results indicate that the transfer of resistance gene between isolates might have occurred, that lead to the development of resistance to all the twelve antibiotics by isolates. The antibiotic resistance genes can spread from one bacterium to another through several mechanisms. Intrinsic resistance is estimated to present a minimal potential for horizontal spread (between different bacterial species), demonstrated for example with the chromosomal vancomycin resistance determinant of the *Lactobacillus rhamnosus* strain GG (Tynkkynen et al., 1998).

The effect of pH played an important role in the antibacterial activity of bacteriocins. The bacteriocin of A3R, A5R and A8R inhibited the growth of common food spoilage bacteria at pH 5. The bacteriocins of these isolates may be useful as biopreservative agents in food industry. LAB are more tolerant to acidic pH and other organisms are inhibited at low pH and most of the LAB thrive best at a pH < 4.5 (Aroutcheva et al., 2001; Linhares et al., 2011; Redondo-Lopez et al., 1990).

The absence of antibacterial activity (absence of zone of inhibition) indicate the degradation of bacteriocins in presence of Proteinase K, suggested the proteinaceous nature of bacteriocin. The similar studies, sensitivity of the extracted bacteriocin to proteolytic enzymes, indicated the proteinaceous nature of bacteriocin (Deraz et al., 2005).

The bacteriocin production varies with the different isolates. Variation in the concentration of was due to the amount of bacteriocin produced by isolates. Studies in our laboratory revealed the fermented green gram batter was a potential source of lactic acid bacteria, produced bacteriocins. Similar studies were performed and tested for bacteriocin production by *Lactobacillus* isolates (Mohankumar and Murugalantha, 2011).

The preservative effect of CP was enhanced in the presence of bacteriocin (BP) at two different concentrations (1% & 4%) in coconut water, increased the preservative effect of CP, indicated bacteriocin as potent biocontrol agent against food spoilage microorganisms. The bacteriocin of A3R isolate could serve as potential biopreservative agent in food industry. Vescovo et al (1995) observed a reduction in high initial bacterial loads of ready-to-use mixed salads on addition of bacteriocin producing LAB. The bacteriocin producing starter cultures are useful for fermentation of sauerkraut or olives to prevent the growth of spoilage organisms (Schillinger et al 1996). This study would help the use of synergistic effects of these natural preservatives in combination with advanced hurdle technologies could result in replacement of chemical preservatives and could allow in maintaining the quality of food.

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