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Isolation and Characterization of Bacteriocin Producing Lactic Acid Bacteria from Fermented Bengal Gram

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ABSTRACT
Bacteriocins are the extracellular proteins produced by the bacteria which inhibit the growth of similar or closely related bacterial strains. Lactic Acid Bacteria (LAB) are predominantly present in the fermented foods produce bacteriocins. In the present study, six strains (three strains each) were isolated from the fermented Bengal gram samples containing with husk and without husk.The organisms isolated were identified as Pediococcussp and Yeast sp.by the biochemical characterization. The bacteriocins produced by these organisms effectively inhibited the growth of E.coli, Klebsiellasp, Staphylococcus aureus, and Pseudomonas aerugenosabut couldn't inhibit the growth of Proteus vulgaris and Bacillus sp. Strains c2 and c3 showed greater activity at low pH. All the strains showed antibacterial activity against Bacillus sp,E.coli, Klebsiellasp, Staphylococcus aureusand Pseudomonas aerugenosabut Proteus vulgaris showed high resistance when bacteriocin was exposed to temperatures 37°C and 80°C.
Keywords: *Antibacterial Activity,Bacteriocin, Lactic Acid Bacteria.*

I. Introduction

The Lactic acid bacteria (LAB) refer to a large group of beneficial bacteria that have similar properties and all produce lactic acid as an end product of the fermentation process. Additional characteristic flavours and aromas are often the result of other products of lactic acid bacteria. LAB prevents the growth of pathogenic bacteria in different eco-systems by production of antimicrobial substance such as lactic acid, acetic acid, diacetyl, and hydrogen peroxide and bacteriocins[1]. Bacteriocin in fermented foods, originally defined asproteinaceous compounds [2,3]. For the first time bacteriocin were discovered by Gratia [4]. Bacteriocins are extracellulary released peptides or protein molecules produced by LAB, with a bactericidal and bacteriostatic mode of action against closely related species.

The antimicrobial activity of LAB plays an important role in the food industry, agriculture and pharmaceutical industry. They are used as bio preservative agent [5]. The inhibitory spectrum of some bacteriocins includes food spoilage and/or food-borne pathogenic microorganisms [6]. However, some of the bacteriocins produced by Gram positive bacteria, which exhibit a much broader spectrum of antagonism. These bacteriocins may act not only against related species, but also against unrelated genera [7]. Bacteriocins are typically considered to be narrow on antibiotics [8]. Bacteriocins may act on cells in different ways to lyse the cell [9]. These compounds cause leakage of ions and other cellular components, and disrupt the proton motive force which results in cell death [10].The competitive removal of essential substrates, the accumulation of D-amino acids, a lowering of oxidation-reduction potential and coaggregation may further restrict undesirable microorganisms. The present study includes, isolation of LAB from fermented Bengal gram, their effect of bactericidal and biopreservative properties.

II. Materials And Methods

2.1. Sample collection and preparation

Bengal gram with husk and without husk were collected and cleaned separately. They were soaked in water for 8hrs so that the grains get soften. The soaked grains was made into batter using electric blender. The two batters were allowed to ferment at room temperature overnight.

2.2. Isolation of organism from fermented bengal gram

One gram of Bengal gram with husk and 1g of Bengal gram without husk were weighed and added into 100 ml of distilled water. After homogenization, serial dilutions were performed up to 10^{-10} and plated on de Man Rogosa and Sharpe (MRS) agar. Three samples of each with husk and without husk are taken. The plates were incubated at 37°C for 24 h. The colonies were then sub cultured in MRS broth twice and plated on MRS agar for its purity.

2.3. Identification of organisms

The cultures were identified according to their morphological, cultural, physiological and biochemical characteristics [11, 12]. The used tests were: Gram reaction; production of catalase, cytochrome oxidase and hydrogen peroxide; acid production from carbohydrates. The HIMEDIA carbohydrate fermentation discs of Fructose, Galactose, Lactose, Maltose, Mannitol and Sucrose were used. The tubes were filled with peptone water, Durham tubes and Methylene Blue (indicator) was added to each tube. The tubes are inoculated with isolated organisms and the respective carbohydrate discs were added, incubated at 37º C for 24 h and observed for acid and gas production. Durham tubes are used to detect the production of gas by isolated organisms. The colour change from blue to yellow indicates the production of acid by isolated organism.

2.4. Preparation of culture supernatants

As bacteriocins are extracellular released proteins, culture supernatants were tested for antibacterial activity. The pure culture isolated from fermented Bengal gram were propagated in MRS broth and incubated at 37°C for 24h.Bacterial cells were separated by centrifugation at 10,000 rpm for 20 min. The supernatant of each sample was collected in to different test tubes separately and is used for further tests.

2.5. Antibacterial activity of culture supernatants

The antibacterial activity of culture supernatants were tested against both gram positive and gram negative organisms such as *Bacillus sp, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiellasp, Proteus vulgaris* by agar well diffusion method.

2.6. Antibiotic sensitivity of isolated strains

The responses of isolated organism to commercially available antibiotic discs were observed on MRS agar at 37°C for 24h. The zone of inhibition (sensitivity) around the discs and absence (resistance) of zone around the discs were noted down.

2.7. Characterization of partially purified bacteriocin

The bacteriocin produced by different strains wasstudied with respect to stability at different temperature and pH.

2.7.1. Effect of heat treatment

The culture supernatant of 1 ml each was taken in eppendorf tube. The water bath was set according to the required temperature using thermometer. The culture supernatant samples were exposed for 1 h, at 37°C and 80°C. The bacteriocin activity was tested after 20 min by agar well diffusion.

2.7.2. Effect of pH sensitivity

The sterile cell free supernatants were adjusted with sterile NaOH or HCl to pH values of 2 and 7, incubated at room temperature for 4 hand analyzed for bacteriocin activity.

2.7.3. Effect of bacteriocin as biopreservative agent

The biopreservative property of bacteriocin of only one Isolate c2 was observed. The bacteriocin 200µl was applied on the surface of fresh Tomato, Capsicum and Brinjal with the help of sterile cotton then left at room temperature and observed for spoilage after seven days.

III. Result And Discussion

3.1. Isolated strains

The five isolated colonies from the fermented bengal gram on MRS media appeared circular, 2 mm in size, creamish white in colour. The isolates c1, c2, c3 (with husk) and c/o1, c/o3 (without out husk) were Gram positive cocci. The isolate c/o2 was spindle shaped and identified as *Yeast species*. The Isolates showed no hemolysis on blood agar.

Table I: General properties of isolated strains

Sample	General properties	Identified organism
c1(Bengal Gram with husk)	Gram positive cocci, Catalase -ve, Oxidase -ve, No hemolysis	*Pediococcus sp*.
c2 (Bengal Gram with husk)	Gram Positive Cocci, Catalase -ve, Oxidase -ve, No hemolysis.	*Pediococcus sp*.
c3(Bengal Gram with husk)	Gram Positive Cocci, Catalase -ve, Oxidase -ve, No hemolysis.	*Pediococcus sp*.
c/o1(Bengal Gram without husk)	Gram Positive Cocci, Catalase -ve, Oxidase -ve, No hemolysis.	*Pediococcus sp*.
c/o2 (Bengal Gram without husk)	Gram Positive, buddingtypecells	*Yeast sp*.
c/o3 (Bengal Gram without husk)	Gram Positive Cocci, Catalase -ve, Oxidase -ve, No hemolysis.	*Pediococcus sp*.

All the isolates showed negative results for catalase and oxidase tests. The isolates c1, c2, c3, c/o1 and c/o3 showed no growth on Mannitol salt agar (MSA) Based on Bergey's Manual of Systematic Bacteriology the strains isolated on the MRS plates were identifies as non-pathpgenic*Pediococcussp* (Table I). The characterization studies of the bacteriocin produced by the six isolated was carried out under aerobic conditions. Screened and characterized 100 strains of bacteriocin producing lactic acid bacteria from traditional fermented foods [19] [13]and Identified 16 lactic acid bacteria from raw and fermented products[14].

3.2. Carbohydrate fermentation

The Table II shows the variation in the sugar fermentation by isolated species. The fermentation of fructose, maltose, mannitol and sucrose were observed by four isolates (c1, c2, c3 and c/o1). The Isolate c/o2 fermented fructose, galactose and sucrose. Maltose and mannitol were fermented by four isolates (c2, c3, c/o1 and c/o2) fermented sucrose. The mannitol was fermented by c3 and maltose was fermented by c/o1. The c1, c2, c3 (with husk) fermented lactose whereas the c/o1, c/o2, c/o3 (without husk) were negative for lactose fermentation. The isolate c/o 3 didn't ferment any of the carbohydrates that were included in the experiment. The lactose was the only carbohydrate that was not fermented by isolates of fermented bengalgram.

Table II: Carbohydrate fermentation test of isolates

Isolates	Fructose	Galactose	Lactose	Maltose	Mannitol	Sucrose
c1	+ve	+ve	-ve	+ve	+ve	+ve
c2	+ve	-ve	-ve	+ve	+ve	+ve
c3	+ve	+ve	-ve	+ve	+ve	+ve
c/o1	+ve	-ve	-ve	+ve	+ve	+ve
c/o3	-ve	-ve	-ve	-ve	-ve	-ve

+ve: Positive (Presence of carbohydrate fermentation),
-ve: Negative (Absence of carbohydrate fermentation).

The bacteriocin producing LAB have attracted significant attention because of their GRAS status and potential use as safe additives for food preservation [15].Bacteriocin producing LAB isolated from fermented foods are one of the best substances for improving the microbiological safety of that particular food as that are adapted to traditional conditions better than those isolated from other sources [16]. The isolates from the fermented Bengal gram batter were tested for the ability to produce lactic acid (Table II). The LAB produces lactic acid as the major end product of the fermentation of carbohydrates and they are the most important bacteria in desirable food fermentations [17].

3.3. Antibacterial activity of bacteriocin against indicator organisms

Table III depicts the antibacterial activity of the isolated strains. The six indicator organisms used for testing antagonistic property of bacteriocin were *Bacillus subtilis, Escherichia coli, Klebsiella sp., Proteus vulgaris, Pseudomonas aeruginosa*and *Staphylococcus aureus*. In the present study, the highest inhibitory activity was observed by bacteriocin of all the six isolates against *Escherichia coli* and no antagonistic activity against *Proteus vulgaris*. The bacteriocin of isolate c/o1 isolate was not able to inhibit S.aureus at 37^{o}C but showed antagonistic activity at 37^{o}C with all indicator strains. At temperature 80^{o}C bacteriocin of c/o1 isolate showed antagonistic activity with all the strains. Inhibition of *E.coli* and *Klebsiellasp.* were observed at 37^{o}C whereas at 80^{o}C only *Klebsiella sp.* was inhibited not *E.coli.*

Table III: Effect of temperature on antibacterial activity of bacteriocins

Zone of inhibition (mm)												
Organism	***B.subtilis***		***E.coli***		***Klebsiella sp.***		***P.aeruginosa***		***S.aureus***		***P.vulgaris***	
Temperature (°C)	**37**	**80**	**37**	**80**	**37**	**80**	**37**	**80**	**37**	**80**	**37**	**80**
Isolates												
c1	-	17	20	-	19	15	21	25	15	14	-	-
c2	15	13	15	-	15	17	25	18	16	10	-	-
c3	12	16	13	-	14	15	-	17	15	-	-	-
c/o1	10	18	20	20	16	13	21	25	-	16	-	-
c/o3	-	15	16	-	16	18	20	22	15	-	-	-

The inhibitory effect demonstrated by isolated strains against indicator organisms indicates the property of antibacterial activity. The MRS medium is better for the cell growth and bacteriocinproduction than any other media [18]. Generally maximum production corresponds with maximum cell concentration. Bacteriocin production by LAB occurs during active growth phase. The high cell yield does not necessarily result in increased bacterioncin activity as several mechanisms can be responsible for the decrease of activity, such as protein aggregation proteolytic degradation by specific and non-specific enzymes and bacteriocin adsorption to producer cells [19].

3.4. Antibacterial activity of bacteriocin of isolates and positive controls (Milk and Sporolac powder)

The antibacterial activity (Figure 1) could not be attributed to the production of organic acids and hydrogen peroxide. By using MRS medium, which contains low glucose (0.2%) the carbon source was reduced so that the LAB cannot produce organic acids to the level that affects the growth of the other bacteria.

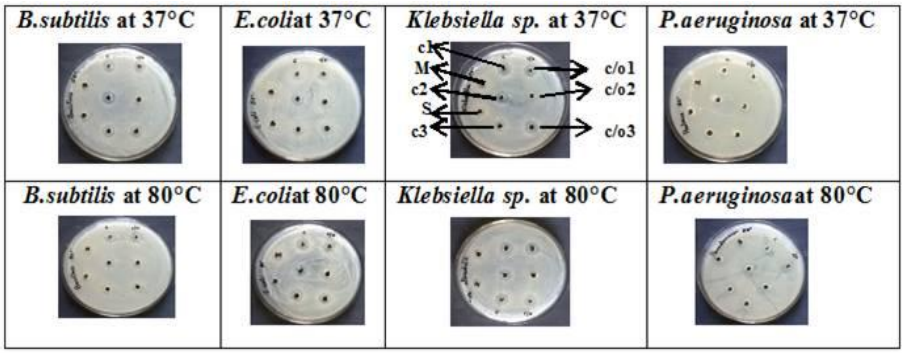

Figure 1: Antibacterial activity of bacteriocinof isolates c1, c2, c3, c/o1, c/o3, M (Milk: Positive control), S (Sporolac Powder: Positive control)

Under aerobic conditions LAB cannot produce hydrogen peroxide to inhibit the growth of indicator organisms.The results indicate that all isolates showed antibacterial effect against *Bacillus subtilis, Escherichia coli, Klebsiella sp., Pseudomonas aeruginosa*and *Staphylococcus aureus*(Picture not included), confirming the inhibition was due to bacteriocin[20, 21]. The growth of *Proteus vulgaris* was not inhibited by bacteriocin of all isolates, indicating its resistant nature towards bacteriocin (Picture not included).

3.5. Antagonistic activity of bacteriocin at pH 2 and pH 7

The Table IV depicts the antagonistic activity of the bacteriocin produced by the six isolates at pH 2 and 7. All the bacteriocins were active and stable at pH 2 against *B.subtilis*, *E. coli*, *Klebsiella sp.*, *P. aeruginosa* and *S.aureus* and *P.vulgaris*. The maximum activity was observed in the bacteriocin produced by isolate c1 and the least activity by isolate c/o1.

Table IV: Effect of pH on antibacterial activity of bacteriocin

Zone of inhibition (mm)												
Organism	***B.subtilis***		***E.coli***		***Klebsiella sp.***		***P.aeruginosa***		***S.aureus***		***P.vulgaris***	
pH	**2**	**7**	**2**	**7**	**2**	**7**	**2**	**7**	**2**	**7**	**2**	**7**
Isolates												
c1	19	15	30	-	32	-	32	-	28	-	30	-
c2	20	20	20	25	29	30	29	25	20	-	20	-
c3	19	20	19	19	25	16	26	21	22	-	24	-
c/o1	15	30	25	-	24	-	-	-	18	-	19	-
c/o2	20	40	24	-	27	12	30	-	24	-	28	-
c/o3	15	14	25	-	24	-	34	-	30	-	29	-

The bacteriocin of isolates c2 and c3 inhibited the growth of all indicator organisms at pH 2 and pH 7, indicating its stability at acidic and neutral pH.A study related to the pH dependent activity of bacteriocin produced by LAB showed that the majority of strains have the highest microbial activity at pH range of 2.0 - 4.0 [22].

3.6. Antibiotic sensitivity of isolates

The isolated strains (Table V) were tested against commercially available antibiotic discs of Ampicillin, Penicillin, Streptomycin and Tetracycline. The Isolates c1, c2, c3 and c/o1 were sensitive to against Ampicillin and Tetracycline and resistant against Penicillin and Streptomycin. The isolates c/o2 and c/o3 were resistant against to all four antibiotics.

Table V: Antibiotic sensitivity test of isolates against four antibiotics

	Antibiotics			
Isolate	**Ampicillin**	**Penicillin**	**Streptomycin**	**Tetracycline**
c1	+ve	-ve	-ve	+ve
c2	+ve	-ve	-ve	+ve
c3	+ve	-ve	-ve	+ve
c/o1	+ve	-ve	-ve	+ve
c/o2	-ve	-ve	-ve	-ve
c/o3	-ve	-ve	-ve	-ve

+ve: Positive (Sensitive); -ve: Negative (Resistant)

The performance of antimicrobial susceptibility testing by the clinical microbiology laboratory is important to confirm susceptibility to chosen empirical antimicrobial agents, or to detect resistance in individual bacterial isolates. Susceptibility testing of individual isolates is important with species that may possess acquired resistance mechanisms [23].

3.7. Bio preservative property of bacteriocin

The partially purified bacteriocin from only one isolate c2 was tested for preservative effect. The bacteriocin of isolate c2 applied topically on the surface of Tomato, Capsicum, Brinjaland observed for a period of one week. The samples without the application of bacteriocin got spoiled within a period of 2-3 days whereas the samples with the bacteriocin application were still fresh for seven days. This experiment revealed that the bacteriocins application prevented the growth of spoilage bacteria, indicating the biopreservative property of bacteriocin. A potentially novel pediocin NV5 was found active against some species of *Enterococcus*, *Leconostoc*, *Staphylococcus*, many of which are associated with food spoilage and food related health hazards [24].

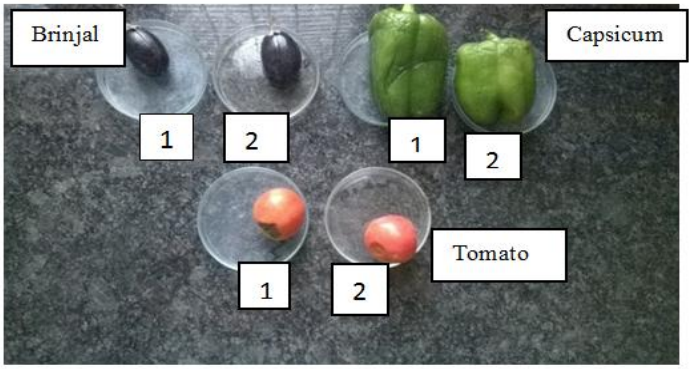

Figure 2: Bio preservative property of bacteriocin of isolate c2
Brinjal 1, Capsicum 1, Tomato 1**:** No topical application of bacteriocin (Spoiled).
Brinjal2, Capsicum 2, Tomato 2**:** Topical application of bacteriocin (Healthy).

IV. Conclusion

Inconclusion Lacticacidbacteria isolates from fermented Bengal gram batter had good antimicrobial activity against foodborne pathogens. The bacteriocin of two isolates was active at pH 2 and pH 7. The topical application of bacteriocin of isolate c2 showed preservative effect. These studies highlight the possibility that these bacteriocins and LAB will be notified of great interest in terms of antimicrobial compounds in further research.

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