International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2015): 6.391

Antifungal Activity of Bacteriocin Produced by Lactic Acid Bacteria from Fermented Green Gram

B.Aruna[1], G. Sai Jeevana Madhuri[2]

[1,2]Department of Microbiology, St. Francis College for Women, Begumpet, Hyderabad-500016, India

Abstract: ***The biopreservative approach of foods using bacteriocinogeniclactic acid bacteria (LAB) is an innovative approach. LAB is predominantly present in the fermented food are well known for the production of bacteriocin. The bacteriocins are proteins produced by bacteria which inhibit the growth of similar or closely related bacterial strains. They are considered as "food-grade" organisms, have been isolated from grains, dairy and meat products, fermenting vegetables, and the mucosal surfaces of animals. These microorganisms present in fermented foods are beneficial and used as probiotics. They are often called as "GRAS" organisms (Generally Regarded as Safe). The main objective of this study was to isolate bacteriocin producing LAB from fermented green gram and identify their antifungal activity. The three isolates G1, G2, G3(green gram with husk) and three isolates GA, GB, GC (green gram without husk) were isolated from fermented green gram. The isolates were identified as Pediococcus sp. These isolates were inoculated into MRS broth for the production of bacteriocin. Indicator strains (fungi) were isolated from spoiled fruit (Papaya) and spoiled vegetables (Tomato, Brinjal, and Onion).The bacteriocin of isolates was tested for their antifungal activity against indicator organisms. The study demonstrated the inhibition of fungal growth by bacteriocins, thus indicating the use of bacteriocinsas potent antifungal substance and biopreservative agent.***

Keywords: Antifungal activity, Bacteriocin, Lactic Acid Bacteria

1. Introduction

Fermentation is the process of digesting certain substances that leads to chemical conversion of organic substances into simpler compounds. Fermentation in food processing is the conversion of carbohydrates to alcohols and carbon dioxide or organic acids using yeasts, bacteria. Fermentation is one of the oldest forms of food preservation in the world. The technology of fermentation began with sweet substances in different parts of the world. Lactic acid bacteria (LAB) are the principle organisms involved in the manufacture of cheese, yogurt, buttermilk and many other dairy products. Lactic acid bacteria prevent the growth of pathogenic bacteria in different ecosystems by production of antimicrobial substances such as lactic acid, acetic acid, diacetyl, hydrogen peroxide and bacteriocins[1].Lactic acid bacteria belong to non-pathogenic genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*and*Leuconostoc*[2]. LAB is the most common microbes employed as probiotics. Most probiotic strains belong to the genus *Lactobacillus.* Probiotics have been evaluated in research studies in animals and humans with respect to antibiotic-associated diarrhea, travellers' diarrhea, pediatric diarrhea, inflammatory bowel disease and irritable bowel syndrome. In the future, probiotics possibly will be used for gastrointestinal diseases, vaginosis, as delivery systems for vaccines, immunoglobulins, and other therapies [3].

These bacteriocins may act not only against related species, but also against unrelated genera [4]. The inhibitory spectrum of bacteriocins includes food-borne pathogenic microorganisms [5].Fresh vegetables and fruits harbor various microorganisms, capable of growing at refrigeration and room temperature. Moreover, it is also tolerate to acidic pH and salt concentrations up to 10% [6, 7]. It is important to seek biopreservatives that control both spoilage and pathogenic microorganisms. Since the isolation and screening of microorganisms from natural sources has always been the most powerful means for obtaining useful and genetically stable strains for industrially important products [8],in the present day, we isolated and identified bacteriocinogenic LAB from fermented green gram (with and without husk) and then further evaluated their antifungal effects invitro against food spoilage fungi (indicator organisms) isolated from spoiled fruit (papaya) and spoiled vegetables (tomato, brinjal and onion).

2. Materials and Methods

2.1. Sample collection and preparation

Green grams (with husk and without husk) were collected and cleaned. They were soaked in water for 8 hr so that they become softened were made into batter using an electric blender. The batter was allowed to ferment overnight at room temperature.

2.2. Isolation of LAB from fermented green gram batter

One gram of fermented green gram batter with husk and 1g of green gram batter without husk were weighed and added separately into 100 ml of distilled water, serial dilutions were performed up to 10^{-10} and 100 ul from each dilution was plated on de Man Rogosa and Sharpe (MRS) agar. The plates were incubated at 37°C for 24 hr. The isolated colonies were plated on MRS agar and maintained as pure cultures.

2.3. Identification of isolated organism

The isolated organisms were identified by Gram staining, production of catalase, cytochrome oxidase and hydrogen peroxide [9].The isolates were also identified for carbohydrate fermentation by using HI-MEDIA

carbohydrate fermentation discs of Maltose, Sucrose, Lactose, Fructose and Galactose.

2.4. Carbohydrate fermentation test

To the tubes containing peptone water the Durham tubes and Methylene Blue (indicator) were added. The tubes were inoculated with isolated organisms and the respective carbohydrate discs (Maltose, Sucrose, Lactose, Fructose and Galactose) were added. The tubes were incubated at 37ºC for 24 hr 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.

2.5. Antibiotic sensitivity test

The isolates were screened for susceptibility to antibiotics by using commercially available antibiotic disc (HI-MEDIA) method. Isolates were grown in MRS broth, incubated for 16-18 hr at 37^{o}C. The active culture (0.1 mL) was spread on MRS agar plates, antibiotic discs were placed on to agar medium and plates were incubated for 24 h at 37^{o}C. The plates were examined for inhibition zones, which are then measured (mm). The zone of inhibition indicated the susceptibility and absence of zone of inhibition indicated the resistance to the antibiotic.

2.6. Preparation of culture supernatant

As bacteriocins are extracellular released proteins, culture supernatants were tested for antifungal activity. The pure culture isolated from fermented green gram batter were propagated in MRS broth and incubated at 37ºC for 48 hr. The bacterial cells were separated by centrifugation at 10,000 rpm for 20 min; pH of the filtrate was adjusted to pH 7 using 1M NaOHto avoid organic acid effect. The supernatant was filtered and was used to determine the antifungal activity of bacteriocins.

2.7. Isolation of fungi (Indicator organisms) from spoiled fruit and vegetables

The spoiled fruit (Papaya) and spoiled vegetables (Tomato, Brinjal, and Onion) were brought to laboratory. Spores from spoiled fruit and vegetables were inoculated on Sabouraud agar (SA), incubated at room temperature (27ºC) for 3-5 days and observed for the growth of fungi.

2.8. Antifungal activity of bacteriocins

Two types of media (SA - Sabouraud agar and CDB - CzapekDox Broth) were chosen to observe antifungal activity of bacteriocins. The SA was used for agar well diffusion method and CBD was used for tube method (turbidimetric method).

2.8.1. Plate method-Agar well diffusion method

The antifungal activity of bacteriocin was tested against indicator organisms (fungi,isolated from spoiled fruit and vegetables) by the agar well diffusion method on SA. The spores of indicator organisms were plated on SA, the holes of 4 mm were made in the SA using a cork borer. To the wells the culture supernatant containing crude bacteriocin of 100µLof all isolates was added. The plates were incubated at room temperature (27^{o}C) for 3-5 days.

2.8.2. Tube method-Tubidimetricmethod

The spores from the SA were inoculated into CDB (500 ul of broth was added to 1.5 mL eppendorf tubes) incubated for 3-5 days at room temperature (27ºC) in presence of bacteriocinof six isolates (G1, G2, G3, GA, GB, GC)with two different concentrations(200 ul and 700 ul)and observed for fungal growth (presence of mycelium and spores).

3. Results and Discussion

3.1 General Properties of Isolates

The bacteriocin producing lactic acid bacteria were isolated from fermented green gram batter with husk and without husk (Table 1). The three isolates were from green gram with husk (G1, G2, G3) and three (GA, GB, GC) from green gram without husk. The colony morphology of six isolates on MRS agar was small, round, colorless and pinpoint. The isolates were gram positive cocci, catalase negative, oxidase negative, coagulase negative, no hemolysis on blood agar plates. Based on their physiological and biochemical characteristics they were identified as *Pediococcus sp*. Among bacteria associated with fermented foods and alcoholic beverages, lactic acid bacteria (LAB) species of *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Weissella*, etc. are widely present in many fermented foods and beverages [10, 11].

Table 1: Isolation and Identification of organisms

Sample	*Properties*	*Organism identified*
G1 Green gram (with husk)	Gram positive cocci, catalase negative, oxidase negative, coagulase negative, no hemolysis on blood agar	*Pediococcussp*
G2 Green gram (with husk)	Gram positive cocci, catalase negative, oxidase negative, coagulase negative, no hemolysis on blood agar	*Pediococcussp*
G3 Green gram (with husk)	Gram positive cocci, catalase negative, oxidase negative, coagulase negative, no hemolysis on blood agar	*Pediococcussp*
GA Green gram (without husk)	Gram positive cocci, catalase negative, oxidase negative, coagulase negative, no hemolysis on blood agar	*Pediococcussp*
GB Green gram (without husk)	Gram positive cocci, catalase negative, oxidase negative, coagulase negative, no hemolysis on blood agar	*Pediococcussp*
GC Green gram (without husk)	Gram positive cocci, catalase negative,oxidase negative, coagulase negative,no hemolysis on blood agar	*Pediococcussp*

3.2 Fermentation of carbohydrates by isolates

The table 2 summarizes the fermentation of carbohydrates by isolates with the production of acid, indicated by color change of medium from blue to yellow and none of the isolates produced gas. Only one isolate GA didn't ferment maltose. All the isolates fermented Sucrose, Lactose, Fructose and Galactose. The isolates G1, G2, G3 GB and GC fermented maltose and only one isolate GA couldn't ferment maltose. There was no production of gas by isolates. The API 50 CHL carbohydrates fermentative profile of *Lb. paracasei ssp. paracasei 2* did not agree with the findings published [12] in that all the strains in this study showed the ability to produce acid from dulcitol and inositol.

Table 2: Carbohydrate fermentation pattern of isolates

Sample	Maltose	Sucrose	Lactose	Fructose	Galactose	Mannitol
G1	+	+	+	+	+	+
G2	+	+	+	+	+	+
G3	+	+	+	+	+	+
GA	-	+	+	+	+	+
GB	+	+	+	+	+	+
GC	+	+	+	+	+	+

(+):Presence of fermentation, **(-)**: Absence of fermentation

3.3 Antibiotic sensitivity tests of isolates

The table 3 shows phenotypic antimicrobial resistance of a strain to each antibiotic, determined by using antibiotic discs (HI-MEDIA). The isolates G3 and GB were sensitive to the antibiotics of Cotrimoxazole, Penicillin, Azithromycin, Ofloxacin, Metronidazole, Linezolid, Clindamycin, Chloramphenicol, Oxacillin, Ampicillin, Clarithromycin, Gentamicin, Amoxicillin-Clavulanate, Vancomycin, Cefepime, Amikacin, Novobiocin, Erythromycin and Tetracycline. The isolate G1 was resistant to Pencillin, Ofloxacin, Linezolid, Clindamycin and Vancomycin. The isolates G2 and GA showed resistance to only one antibiotic vancomycin. The GC was the only isolate showed resistance to only one antibiotic, Cefepime. The non-permeabilityof the lactobacilli cell wall is the main reason for their resistance, but several nonspecific mechanisms could cause differences among strains within the same species [13, 14].

Table 3: Antibiotic sensitivity tests of isolates

Antibiotic	G1	G2	G3	GA	GB	GC
Cotrimoxazole	+	+	+	+	+	+
Penicillin	-	+	+	+	+	+
Azithromycin	+	+	+	+	+	+
Ofloxacin	-	+	+	+	+	+
Metronidazole	+	+	+	+	+	+
Linezolid	-	+	+	+	+	+
Clindamycin	-	+	+	+	+	+
Chloramphenicol	+	+	+	+	+	+
Oxacillin	+	+	+	+	+	+
Ampicillin	+	+	+	+	+	+
Clarithromycin	+	+	+	+	+	+
Gentamicin	+	+	+	+	+	+
Amoxicillin-Clavulanate	+	+	+	+	+	+
Vancomycin	-	-	+	-	+	+
Cefepime	+	+	+	+	+	-
Amikacin	+	+	+	+	+	+
Novobiocin	+	+	+	+	+	+
Erythromycin	+	+	+	+	+	+
Tetracycline	+	+	+	+	+	+

(+): Sensitive, (-): Resistant

34 Isolation of fungi (indicator organisms) from spoiled fruit and vegetables

The spoilage of fruits and vegetables by fungi is the primary source of spoilage agent. The table 4and Figures 1, 2, 3 and 4summarizes the fungal species (Indicator organisms: *Aspergillusflavus, Fusarium*sp and *Aspergillusniger)* isolated from spoiled fruit (Papaya) and spoiled vegetables (Tomato, Brinjal, Onion) using Sabouraud agar. Fungal spoilage of food is a world-wide phenomenon. Fungal spoilage of date-palm fruits of Saudi Arabia has been reported [15].

Table 4: Isolation of fungi (indicator organisms) from spoiled fruit and spoiled vegetables

Source (Spoiled fruit and vegetables)	Fungi (indicator organisms) and picture indicating colony morphology
Papaya (F)	**Figure 1:** *Aspergillusflavus* 
Tomato (V)	**Figure 2:** *Fusarium*sp. 
Brinjaln (V)	**Figure 3:** *Fusarium*sp. 
Onion (V)	**Figure 4:** *Aspergillusniger* 

F: Fruit, V: Vegetable

3.5 Antifungal activity of bacteriocin by Plate method (Agar well diffusion method)

There were no satisfactory results (no inhibitory zones) after a period of incubation of indicator strains (fungi) with bacteriocins(of all six isolates) for 3-5 daysat room temperature (27^{o}C)by the agar well diffusion method (Figure 5). There was no antifungal activity of bacteriocins by plate method (agar well diffusion method),this could be due to absence of diffusion of bacteriocins in the solid media.

Figure 5: Antifungal activityof bacteriocins by agar well diffusion against indicator organisms - No inhibitory zones

This attributed to identify the influence of fungal growth by bacteriocins in presence of liquid medium. The growth of toxigenic fungi of cereals can be restricted by LAB *in vitro,*

achieved an inhibition level of 40% in 22 of 104 combinations of LAB and *P. verrucosum*[16].

3.6. Antifungal activity of bacteriocin by tube method (Tubidimetric method)

The table 5 shows the antifungal activity of bacteriocins against the indicator organisms (fungal species) isolated from spoiled fruit(papaya) and spoiled vegetables (tomato, brinjal and onion). The growth of indicator organisms was observed withbacteriocinat two different concentrations,200 ul and 700 ul. *Aspergillusflavus*(isolated from papaya) and *Fusariumsp*(isolated frombrinjal) could grow (very little myceliawere observed) in presence of 200 ul of bacteriocin butcould not grow (no mycelia) in presence 700 ul of bacteriocin of G1, G3, GA and GC. The200 ul of bacteriocin of two isolates G2 and GB could inhibit the growth of all four fungal species. *Fusariumsp*(isolated from tomato) and *Aspergillusniger*(isolated from onion)were sensitive to bacteriocin of all isolates. The sensitivity of fungal species to bacteriocinwas found to be effective in liquid medium than in a solid medium. This could be due to greater diffusion of bacteriocins in liquid media than in solid medium and high possibility of direct contact of spores with bacteriocins in liquid medium. The AF (Antifungal) effects of *Lactobacillus acidophilus*,inhibited the growth of*Candida albicans*[17].

Table 5: Antifungal activity of bacteriocin at two different concentrations (200 µl and 700 ul) against indicator organisms

F	G1		G2		G3		GA		GB		GC	
	C(ul)		C(ul)		C (ul)		C(ul)		C (ul)		C (ul)	
	a	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
Af (P)	m+	-	-	-	m+	-	m+	-	-	-	m+	-
Fs(T)	-	-	-	-	-	-	-	-	-	-	-	-
Fs (B)	m+	-	-	-	m+	-	m+	-	-	-	m+	-
An (O)	-	-	-	-	-	-	-	-	-	-	-	-

(m+): Presence of very little mycelia, (-): No fungal growth,F: Fungi (Indicator organisms),C:Concentration ofbacteriocin (ul), *Af:Aspergillusflavus*, P: Papaya, *Fs:Fusarium sp.*,T: Tomato, B: Brinjal, *An:Aspergillusniger*, O: Onion,a:200 ul of bacteriocin, b: 700 ul of bacteriocin.

4. Conclusion

This study demonstrated that there was germination of fungal spores with low concentration of bacteriocin but no germination of spores(no fungal growth)in the presence of high concentration of bacteriocin. The bacteriocin of two isolates could inhibit the fungal growth at low concentration, thus indicating the use of bacteriocins as antifungal and biopreservative agents. The fungal spores were resistant to the bacteriocins in solid media. The inhibition of fungal spore germination (fungal growth) was detectable in the liquid cultures. The results of tube method (turbidimetricmethod), confirmed the effectiveness of bacteriocins as antifungal products and potent biopreservative agents.

References

[1] Reid, G. (2001). Probiotic agents to protect the urogenital tract against infection.*Am. J. Clin.Nutr.***73**: 437S-443S.
[2] Sonomoto, K. and Yokota, A (editor). (2011). Lactic Acid Bacteria and Bifidobacteria: Current Progress in Advanced Research. Caister Academic Press.
[3] Tannock, G (editor). (2005). Probiotics and Prebiotics: Scientific Aspects (1st Ed.) Caister Academic Press.
[4] Anne K. Vidaver., Mary L. Mathys., Mary E. Thomas. and Max L. Schuster.(1972). Bacteriocins of the phytopathogens*Pseudomonas syringae, P.glycinea and P. gtycinca, and P. phaseolicola*.*Canadian Journal of Microbiology*. **18**(6): 705-713.
[5] Schillinger, U., R. Geisen, R. and Holzapfel, W, H. **(**1996). Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods.*Trends Food Sci. Technol*.**7:**158-164.
[6] Tasara T. and Stephan, R. (2006). Review cold stress tolerance of Listeria monocytogenes: A review of molecular adaptive mechanisms and food safety implications. *J Food Protection*. **69**:1473-1484.
[7] Vescovo, M., Orsi, C., Scolari, G., Torriani, S. (1995). Inhibitory effect of selected lactic acid bacteria on microflora associatedwithready-to-use vegetables. *Letters in Applied Microbiol*.**21**:121-125.
[8] Ibourahema, C., Dauphin, R.D., Jacqueline, D., Thonart, P. (2008). Characterization of lactic acid bacteria isolated from poultryfarms in Senegal. *African J Biotechnol*.**7**:2006-2012.
[9] Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. and Williams, S.T. (1994). Bergey's Manual of Determinative Bacteriol. 9th Edn., William and Wilkins, USA.
[10] Axelsson, L., Rud, I., Naterstad, K., Blom, H., Renckens, B., Boekhorst J., Kleerebezem, M., van Hijum, S., Siezen, R.J. (2012). Genome sequence of the naturally plasmid-*free Lactobacillus plantarum*strain NC8 (CCUG 61730).*J. Bacteriol*.**194**: 2391-2392.
[11] Holzapfel, W. H. and Wood, B. J. B. (2014). Lactic Acid Bacteria: Biodiversity and Taxonomy.(New York, NY: Wiley-Blackwell), 632.
[12] Charteris, W.P., Kelly, P.M., Morelli, L. and Collins J.K. (2002). Edible table (bio) spreadcontaining potentially probiotic lactobacillus and bifidobacterium species. *International Journal of Dairy Technology*.**55**(1): 44-56.
[13] Condon, S. (1983). Aerobic metabolism of lactic acid bacteria. *Ir. J. Food Sci. Technol*. **7**: 15- 25.
[14] Putman, M., Van Veen, H.W., Degener, J.E., Konings, W.N.(2001). The lactococcal secondary multidrug transporter LmrP confers resistance to lincosamides, macrolides, streptogramins and tetracyclines. *Microbiology*.**147**: 2873-2880.
[15] Al-Sheikh, H. (2009). Date-Palm fruit spoilage and seed-borne fungi of Saudi Arabia.*Res J.Microbiol*.**4**: 208-213.
[16] Vanne, L., Kleemola, T. and Haikara, A. (2000). Microbial assessment and quality evaluation of ogi during spoilage.*World J. Microbiol.Biotechnol*.**18**: 731-737.

[17] Collins, E.B. and Hardt, P. (1980). Inhibition of *Candida albicans* by *Lactobacillus acidophilus*.*J. Dairy Sci*. **63**: 830-832.

Author Profile

Dr.B.Aruna, Assistant Professor, Department of Microbiology, St. Francis College for Women, Begumpet, Hyderabad 500016, Telangana State, India.

G. SaiJeevanaMadhuri,received M.Sc. degree in Microbiology from St. Francis College for Women,Begumpet, Hyderabad. In 2014.