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Isolation and Identification of a Bacteriocinic Substance Producing Bacteria from Various Sources

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A B S T R A C T

Bacteriocins are low molecular proteinaceous antimicrobial compounds secreted by bacteria to inhibit the growth of similar or closely related bacterial strains. In the present study, an attempt was made to isolate bacteria from various sources and screen for bacteriocinic activity. A total of 609 bacteria were isolated from different sources out of which 303 gram positive bacteria were screened for antimicrobial activity by primary streak method. Among these, 51 positive isolates were studied for colony and cell morphology which exhibited good antimicrobial activity. Thirty two isolates were streamlined for determination of bacteriocinic activity by agar well diffusion assay against four pathogens. Further three isolates IB23, AH4 and BrMk4 revealed significantly better antimicrobial activity against *S. aureus, P. aeruginosa* and *E. coli*. very scarce antimicrobial activity was observed against *L. monocytogenes*. Based on the biochemical tests, the organism was identified to be *Staphylococcus aureus, Bacillus cereus* and *Lactobaccilus* sp. respectively.

Introduction

Bacteriocins are generally ribosomally synthesised low molecular weight antimicrobial peptides that are not lethal to the host cells (Law, 2005). It was first discovered by Gratia in 1925 (Gratia, 2000) and they find their application in gastro-intestinal disorders, biotechnological, food and agro-industries (Jack *et al*., 1995). The antimicrobial effect of bacteriocins (Sahu *et al*., 2008) have a potential use as natural food preservatives (Riley, 2009). Prevention of spoilage and pathogenic microorganisms will be more efficient if bacteriocinic activity is increased. Bacteriocins of Gram-positive bacteria are abundantly found and are more diverse than those found in Gram - negative bacteria and they resemble many of the antimicrobial peptides produced by eukaryotes. They are generally non lethal cationic, amphiphilic and membrane-permeabilizing peptides (Sahl *et al*., 1998). They can provide both broad-spectrum killing of many microbes or can target on individual bacterial species.

Besides these applications, they can also be used in combination with other antibiotics for therapeutic use which finds $22 to $24

billion globally at 2-3% increase per annum (Malini Maria, 2012). Their use in food and dairy industry lies in preservation by increasing the shelf- life of the products and are also used as additives, flavour enhancers which replaces harmful chemicals. They also find their application in livestock by feeding on bacteriocin producing bacteria and as probiotics in aquaculture.

Materials and Methods

Isolation of the Culture

A total of 51 different samples, as shown in table 1, were collected in sterile containers. All samples were appropriately labelled and transported to the lab and stored at 10°C for further investigations. Different procedures were followed for different samples based on the load of the microbes as shown in table 1. Serial dilutions of the collected sample were carried out and 0.1 ml of each diluents was transferred to 0.9 ml of sterile distilled water.

Correspondingly dilutions were prepared from 10^{-2} to 10^{-4} according to the sample. The dilution of 10^{-3} and 10^{-4} were used for isolation of bacteria. The inoculum was spread on Nutrient agar (NA) plates and the plates were incubated at 37 °C for 24 hours. Morphological appearances of the inoculated plates were observed after 24 hours of incubation (Fig 1.) and distinct colonies were sub-cultured to obtain pure isolates which were then subcultured on NA slants.

The pure bacterial isolates (Fig 2.) were further identified by microscopic examination after Gram staining. The Colonies which appeared as purple blue i.e. Gram positive upon microscopic observations were subcultured on Nutrient agar plates and preserved at 4°C in the refrigerator for further experiments.

Preliminary Screening of Antimicrobial activity

The pure isolates were further screened for bacteriocinic activity by single streak method against 4 pathogenic bacteria procured from Culture collection centers (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC- 27853, *Staphylococcus aureus* ATCC 13709, and *Listeria monocytogenes* MTCC 1143) as shown in Table 2. Mueller Hinton Agar medium was prepared and aseptically poured into Petriplates. After solidification, lawn culture of indicator microorganisms were made on the agar surface by using sterile cotton swabs. The plates were incubated for 15 minutes in room temperature inside the laminar air flow. After incubation the isolated cultures were streaked in single line using a sterile inoculating loop. The plates were incubated at 37°C for 24 hours (Fig 3.). After 24 hours of incubation period, microorganisms displaying clear zones of inhibition against the pathogens were observed.

Secondary Screening of Antimicrobial Activity

Antimicrobial activity was analysed against various pathogens mentioned above by agar well diffusion method. The pathogens were incubated in Brain-Heart infusion broth at 37°C for different hours and adjusted according to 0.5 McFarland standards was used. The pathogens were lawn cultured using sterile cotton swab. Agar well was made using sterile cork borer and 50µl of isolate supernatants grown in MRS media (centrifuged at 10,000 g for 15 minutes) was incorporated into the wells and incubated for 24 hours at 37°C. After incubation, zone of inhibition was measured (in mm) as shown in table 2. (Fig 4.).

Table.1 Cultural characters of the isolated bacterial colonies from various sources

Sl .No.	Samples	Dilu-tion	No of Colonies	Form/ Shape	Size	Elevation	Surface/ Texture	Margin	Colour	Gram Stain
1	Curd nandini	10^{-3}	11	Spreading, Irregular	Large	Umbonate	Dry	Undulate	Creamy Off white	Positive rod
2	Curd neighbor	10^{-3}	22	Irregular	Moderate	Umbonate	Rough	Wavy	white	Positive rod
3	Curd NDRI	10^{-3}	15	Irregular	Moderate	Convex	dull	Undulate	off white	Positive rod
4	Curd Tirumala	10^{-3}	24	Irregular	Moderate	Flat	Dry	Lobate	White	Positive rod
5	Curd nestle	10^{-4}	7	Circular	Small	Umbonate	Rough	Undulate	Creamy / offwhite	Positive rod
6	Curd nilgiris	10^{-4}	9	Filamentous	Large	Flat	Rough	Undulate	white	Positive rod
7	Curd Amul	10^{-4}	7	Irregular	Moderate	Convex	dull	Undulate	Cream	Positive rod
8	Idly Batter sagar hotel	10^{-3}	14	Circular	Small	Flat	Rough	Wavy	Creamy / off white	Positive rod
9	Idly Batter Srinivas Darshini	10^{-3}	12	Irregular	Moderate	Flat	Rough	Undulate	White	Positive rod
10	Idly Batter megha Sagar	10^{-3}	22	Circular	Small	Flat	Dry	Lobate	Creamy Off white	Positive rod
11	Idly B	10^{-3}	22	Spreading, Irregular	Large	Umbonate	dull	Undulate	off white	Positive rod
12	Idly Batter	10^{-4}	15	Irregular	Moderate	Umbonate	dull	Undulate	off white	Positive rod
13	Idly Batter suma	10^{-4}	13	Circular	Small	Flat	Rough	Undulate	Creamy Off white	Positive rod
14	Raw Milk	10^{-3}	3	Irregular	Moderate	Flat	Rough	Wavy	Whit	Positive rod
15	Raw Milk	10^{-3}	5	Irregular	Moderate	Umbonate	Rough	Lobate	whie	Positive rod
16	Raw Milk	10^{-3}	3	Filamentous	Moderate	Umbonate	Dry	Undulate	whte	Positive rod
17	Raw Milk	10^{-3}	3	Spreading, Irregular	Large	Convex	dull	Wavy	off white	Positive rod
18	Breast Milk	10^{-3}	5	Circular	Small	Flat	Rough	Undulate	Creamy Off white	Positive rod
19	Breast Milk	10^{-3}	1	Spreading, Irregular	Large	Umbonate	Dry	Lobate	Creamy Off white	Positive rod
20	Breast Milk	10^{-4}	5	Filamentous	Large	Umbonate	Rough	Undulate	white	Positive rod

21	Breast Milk	10^{-4}	2	Irregular	Moderate	Convex	dull	Undulate	Cream	Positive rod
22	Breast Milk 1	10^{-4}	2	Circular	Small	Umbonate	Rough	Undulate	Creamy Off white	Positive rod
23	Buttermilk 1	10^{-3}	11	Irregular	Small	Flat	Rough	Lobate	white	Positive rod
24	Buttermilk 2	10^{-3}	09	Irregular	Moderate	Flat	Rough	Undulate	white	Positive rod
25	Buttermilk 3	10^{-3}	05	Irregular	Moderate	Umbonate	Dry	Undulate	White	Positive rod
26	Buttermilk 4	10^{-3}	23	Irregular	Moderate	Convex	Rough	Wavy	Yellowish white	Positive rod
27	Buttermilk 5	10^{-3}	27	Irregular	Moderate	Umbonate	Rough	Undulate	white	Positive rod
28	Buttermilk 6	10^{-4}	21	Filamentous	Large	Flat	Rough	Undulate	white	Positive rod
29	Buttermilk 7	10^{-4}	19	Spreading, Irregular	Moderate	Convex	dull	Undulate	Creamy Off white	Positive rod
30	Buttermilk 8	10^{-4}	21	Circular	Moderate	Flat	Rough	Undulate	Creamy Off white	Positive rod
31	Infant faeces 1	10^{-3}	5	Irregular	Moderate	Umbonate	Dry	Wavy	Cream	Positive rod
32	Infant faeces 2	10^{-3}	6	Irregular	Moderate	Convex	dull	Undulate	Cream	Positive rod
33	Infant faeces 3	10^{-3}	3	Irregular	Moderate	Umbonate	dull	Undulate	off white	Positive rod
34	Infant faeces 4	10^{-3}	4	Circular	Small	Flat	Rough	Undulate	Creamy Off white	Positive rod
35	Infant faeces 5	10^{-4}	5	Spreading, Irregular	Large	Umbonate	Dry	Undulate	Creamy Off white	Positive rod
36	Infant faeces 6	10^{-4}	6	Irregular	Moderate	Flat	Rough	Wavy	white	Positive rod
37	Lassi	10^{-4}	7	Irregular	Moderate	Flat	Dry	Undulate	white	Positive rod
38	Yogurt	10^{-3}	8	Irregular	Moderate	Umbonate	Rough	Lobate	white	Positive rod
39	Yogurt	10^{-3}	9	Irregular	Moderate	Convex	dull	Lobate	off white	Positive rod
40	Ant hill soil 1	10^{-3}	10	Circular	Small	Flat	Dry	Undulate	Creamy Off white	Positive rod
41	Ant hill soil 2	10^{-3}	21	Filamentous	Large	Flat	Rough	Lobate	Whit	Positive rod
42	Ant hill soil 3	10^{-4}	19	Spreading, Irregular	Large	Umbonate	Dry	Undulate	Creamy Off white	Positive rod
43	Ant hill soil 4	10^{-4}	18	Irregular	Moderate	Umbonate	Rough	Wavy	white	Positive rod
44	Dosa batter neighbour 1	10^{-4}	15	Irregular	Moderate	Umbonate	Rough	Lobate	white	Positive rod
45	Dosa batter	10^{-4}	32	Irregular	Moderate	Convex	dull	Lobate	off white	Positive rod

	neighbour 2									
46	Dosa batter neighbour 3	10^{-4}	13	Circular	Small	Flat	Dry	Undulate	Creamy Off white	Positive rod
47	Dosa batter neighbour 4	10^{-4}	23	Filamentous	Large	Flat	Rough	Lobate	White	Positive rod
48	Dosa batter neighbour 5	10^{-4}	15	Spreading, Irregular	Large	Umbonate	Dry	Undulate	Creamy Off white	Positive rod
49	Dosa batter sagar Hotel	10^{-4}	24	Irregular	Moderate	Umbonate	Rough	Wavy	white	Positive rod
50	Fruit juice sample 1	10^{-4}	5	Irregular	Moderate	Convex	dull	Lobate	off white	Positive rod
51	Fruit juice sample 2	10^{-4}	4	Irregular	Moderate	Umbonate	Rough	Lobate	white	Positive rod

Table.2 Antimicrobial activity of isolated bacteria (zone of inhibition in mm)

Sl..No.	Organism code	*S. aureus*	*E. coli*	*L. monocytogenes*
1.	CN 4	5	6	4
2	C N 5	6	5	4
3.	C T 5	6	7	4
4.	**IB 23**	**9**	**7**	**4**
5.	IB 5	7	6	4
6.	BM 4	6	4	4
7.	BM 20	4	7	4
8.	BM 22	6	7	4
9.	BM 24	6	7	4
10.	**AH 4**	**8**	**8**	**5**
11.	AH 7	7	5	4
12.	AH 8	7	4	4
13.	AH 9	6	7	4
14.	AH 12	7	6	4
15.	BrMk 3	7	6	4
16.	**BrMk 4**	**9**	**9**	**5**
17.	BrMk 5	5	7	4
18.	BrMk 6	4	7	4
19.	DB S	7	4	4
20.	DB 5	5	8	4
21.	DB 6	6	7	4
22.	DB 7	5	4	4
23.	DB 8	8	5	4
24.	YG 3	4	6	4
25.	YG 4	5	5	4
26.	Fj 2	4	5	4
27.	Fj 3	4	6	4
28.	RM 2	5	4	4
29.	RM 3	6	4	4
30.	RM 4	5	4	4
31.	RM 5	5	5	4
32.	RM 6	6	5	4

Table.3 Biochemical tests of potential isolates

Biochemical test	**IB23**	**AH4**	**BrMk 4**
Indole	-	-	-
MR	+	-	+
VP	+	-	-
Citrate	+	+	-
Catalase	+	+	-
Starch	+	-	+
Urease	-	-	-
Nitrate reduction	-	-	-
Casein hydrolysis	-	-	-
Glucose fermentation	+	+	+A/G
Fructose Fermentation	+	+/A	+
Mannose Fermentation	+	+/A	+
Galactose Fermentation	_	+/A	+
Lactose fermentation	+	-	+
Gelatin hydrolysis	+	-	-
H_2S Production	+	-	-
Oxidase	-	+	-

Fig.1 Isolation of bacteria from various samples

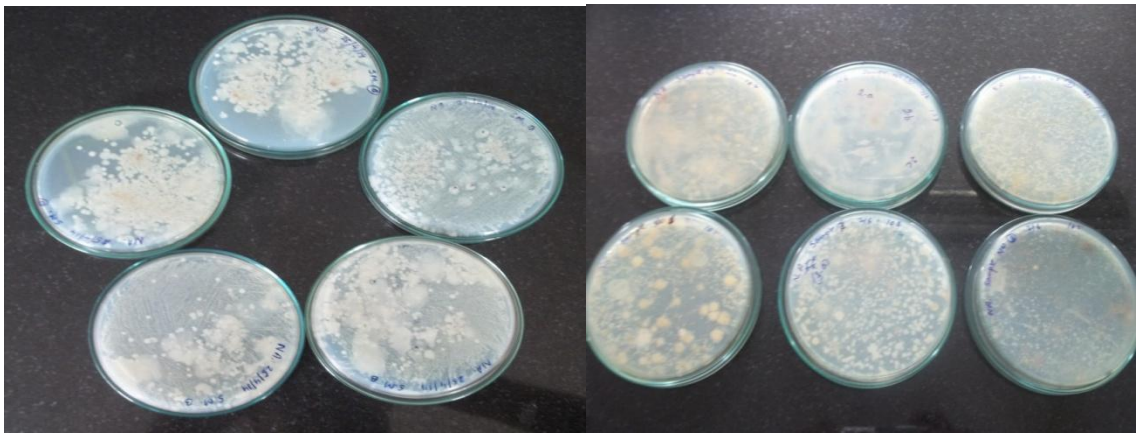


Fig.2 Isolated pure culture

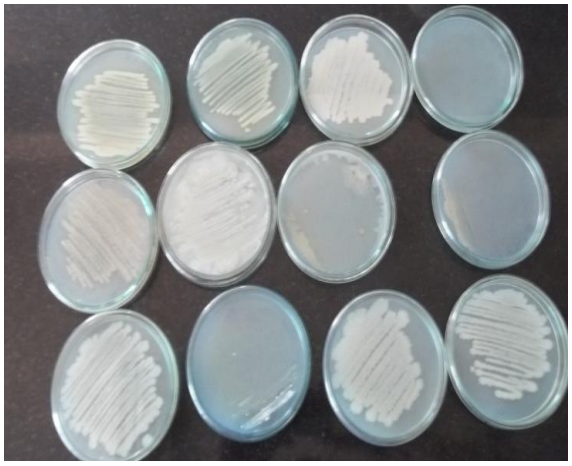


Fig.3 Initial screening of the isolates against a few pathogens

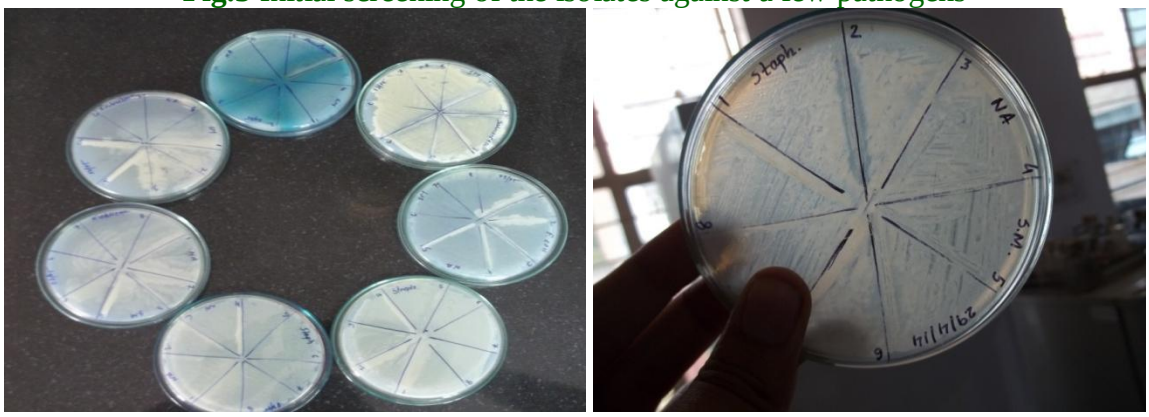
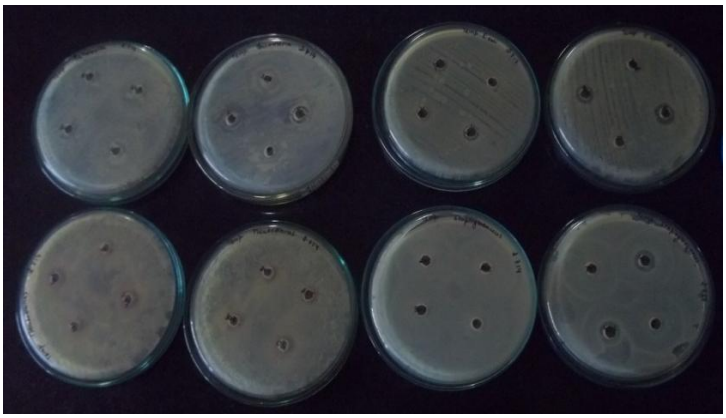


Fig.4 Antimicrobial activity of the isolates



Biochemical Tests

Different Biochemical tests have been performed for identification of potential isolates., (Table 3) according to Bergey's Manual of Bacteriology like indole test, methyl Red test, Vogue's Proskeur's test, citrate test, catalase test, starch, urease,

nitrate reduction test, casein hydrolysis, glucose fermentation test, fructose fermentation test, mannose fermentation test, galactose fermentation test, oxidase test, lactose fermentation test, gelatin hydrolysis test and H_2S production test.

Results and Discussion

A total of 609 bacteria were isolated from various sources, among them 303 gram positive bacteria were screened for antimicrobial activity by primary streak method. Out of these, 51 positive isolates were studied for colony morphology (colour, shape, margin, elevation and surface) and cell morphology (shape, arrangement, and Gram's staining) which exhibited good antimicrobial activity. Further, after secondary screening, 32 positive isolates were selected as they exhibited zone of inhibition against all the four pathogens as shown in the Table 2. On secondary screening three isolates IB23, AH4 and BrMk 4 revealed significantly better antimicrobial activity against *S. aureus, P. aeruginosa* and *E. coli.* However, very scarce antimicrobial activity was observed against *L. monocytogenes.* According to the biochemical tests as shown in Table 3 and the microscopic observation revealed IB23 as *S. aureus,* the organism AH 4 was *Bacillus cereus* and BrMk 4 belonged to *Lactobaccilus* sp.

Ashok *et al*., 2014 reported better antimicrobial activity of isolates from milk and curds against *S .aureus, K .pneumoniae E.coli,* however *P. aeruginosa* was resistant against these isolates. In the present study 32 isolates showed activity against *P. aeruginosa*. Similar to the bacteriocinic activity from *Lactobacillus* sp. isolated from breast milk, Abdulla *et al*., 2014, also quoted antimicrobial compounds from *Lactobacillus acidophilus*. Our study on antimicrobial activity of *Lactobacillus* sp. were similar to the reports of *L. plantarum* of Das *et al*., 2014. However Gaamouche *et al*., 2014, reported better antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes.*

Bacteriocins of Gram-positive bacteria are abundant and diverse than that of Gram - negative bacteria and also differ in ecological and evolutionary aspects. The spectrum of antimicrobial activity is broader in gram-positive bacteria, than that of gram negative species and also less toxic for preservation. In addition, the gram-positive bacteria have relatively higher-molecular-weight, heat-labile bacteriocin-like substances.

In conclusion, the present study on isolation and identification of a bacteriocinic substance producing organisms from various sources provides an overview of the diversity of the ability of microbes to produce antimicrobial substances which could be commercially exploited by the food, dairy, medical industries. It is also evident that the bacteriocin like products of gram-positive bacteria revealed broader antibacterial spectrum and will continue to be an active area of applied research.

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