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

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ABSTRACT

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 Lactobacillus sp. respectively.

Keywords

Bacteriocin, Gram positive, Antimicrobial activity, pathogens, isolates.

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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 gastrointestinal 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 bacteriocin 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                     | Dilution | 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             | Undulate  | White             | Positive rod|
| 5     | Curd nestle                 | $10^{-4}$| 7              | Circular             | Small  | Umbonate  | Rough           | Undulate  | Creamy/Off white  | 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      | White             | Positive rod|
| 15    | Raw Milk                    | $10^{-3}$| 5              | Irregular            | Moderate| Umbonate  | Rough           | Lobate    | white             | Positive rod|
| 16    | Raw Milk                    | $10^{-3}$| 3              | Filamentous          | Moderate| Umbonate  | Dry             | Undulate  | white             | 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|
|   | Sample Type         | Concentration | Number | Shape     | Texture      | Convexity | Undulate | Color                  | Other Characteristics            | Rod Test Result |
|---|---------------------|---------------|--------|-----------|--------------|-----------|----------|------------------------|-------------------------------|----------------|
| 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               | 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 | Convex    | dull     | Undulate | Cream Off white | Positive rod |
| 30| Buttermilk 8       | $10^4$        | 21     | Circular  | Moderate     | Flat      | Rough    | Undulate               | Cream 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               | Cream Off white              | Positive rod   |
| 35| Infant faeces 5    | $10^4$        | 5      | Spreading, Irregular | Large    | Umbonate  | Dry      | Undulate               | Cream 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               | Cream Off white              | Positive rod   |
| 41| Ant hill soil 2    | $10^3$        | 21     | Filamentous| Large        | Flat      | Rough    | Lobate                 | White                        | Positive rod   |
| 42| Ant hill soil 3    | $10^3$        | 19     | Spreading, Irregular | Large    | Umbonate  | Dry      | Undulate               | Cream Off white              | Positive rod   |
| 43| Ant hill soil 4    | $10^4$        | 18     | Irregular | Moderate     | Umbonate  | Rough    | Wavy                   | white                        | Positive rod   |
| 44| Dosa batter        | $10^4$        | 15     | Irregular | Moderate     | Umbonate  | Rough    | Lobate                 | white                        | Positive rod   |
| 45| Dosa batter neighbour 1 | $10^4$  | 32     | Irregular | Moderate     | Convex    | dull     | Lobate                 | off white                    | Positive rod   |
| neighbour | Sample Type | Concentration | Conf. | Shape | Size | Texture | Undulate | Colour | Rod Type |
|-----------|-------------|---------------|-------|-------|------|---------|----------|--------|----------|
| neighbour 2 | Dosa batter neighbour 3 | $10^4$ | 13 | Circular | Small | Flat | Dry | Undulate | Creamy | Off white | Positive rod |
| neighbour 3 | Dosa batter neighbour 3 | $10^4$ | 13 | Circular | Small | Flat | Dry | Undulate | Creamy | Off white | Positive rod |
| neighbour 4 | Dosa batter neighbour 4 | $10^4$ | 23 | Filamentous | Large | Flat | Rough | Lobate | White | Positive rod |
| neighbour 5 | Dosa batter neighbour 5 | $10^4$ | 15 | Spreading, Irregular | Large | Umbonate | Dry | Undulate | Creamy | Off white | Positive rod |
| neighbour 6 | Dosa batter neighbour 6 | $10^4$ | 24 | Irregular | Moderate | Umbonate | Rough | Wavy | White | Positive rod |
| neighbour 7 | Dosa batter neighbour 7 | $10^4$ | 5 | Irregular | Moderate | Convex | dull | Lobate | Off white | Positive rod |
| neighbour 8 | Dosa batter neighbour 8 | $10^4$ | 4 | Irregular | Moderate | Umbonate | Rough | Lobate | White | Positive rod |

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### 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.      | CN 5          | 6         | 5       | 4                |
| 3.      | CT 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        | +    | -   | -      |
| H₂S Production            | +    | -   | -      |
| Oxidase                   | -    | +   | -      |

**Fig.1** Isolation of bacteria from various samples
**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 H₂S 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 Lactobacillus 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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