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In-vitro Characterization and Purification of Bacteriocins Isolated from Probiotic Curd Culture

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Bacteriocins, the polypeptides secreted from probiotics proves to be a better choice of antimicrobials than that of synthetic antibiotics due to their negligible side effects and low cost. The present research focusses on the isolation of bacteriocin producing probiotic strains from homemade curd having good antagonistic properties against acne causing bacteria, Propniobacterium acnes. Bacteriocins are isolated, purified and dried. Morphological and general phenotypic tests are performed for identification of microorganisms. Effect of temperature, pH and bile salts on efficacy of bacteriocins is studied. Antimicrobial activity of bacteriocins is tested against the indicator strain by disc diffusion method. Normal sterile saline is used as a negative control and clindamycin is taken as a positive control. MRS media is used for culturing of microorganisms. Antimicrobial activity is recorded in terms of zone of inhibition in mm.

Results: The isolates obtained from curd belongs to the lactobacillus family is proved by the usual phenotypic tests. Among the seven isolates obtained from curd, bacteriocins produced from isolate 6 shows maximum antimicrobial activity. Bacteriocins are most stable at pH range of 5-6 and temperature of 25-40°C. Activity decreases with increase in concentration of bile salts and completely diminishes at 11% bile salt concentration.

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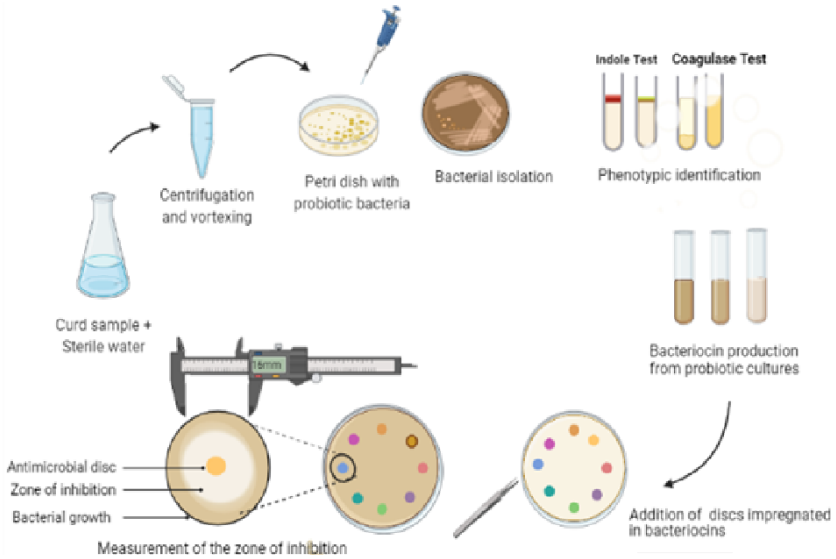
GRAPHICAL ABSTRACT



Keywords: Bacteriocins; probiotics; incubation; inoculation; purification; bile tolerance; pH tolerance; antimicrobial.

1. INTRODUCTION

Bacteriocins are antimicrobial peptides, ribosomally synthesized as an inherent defense mechanism of bacteria discovered 100 years ago [1]. Over 99% of the total bacterial population produces bacteriocins belonging to the abundant class of proteinaceus metabolites with an increasing rate of advantages to human health [2]. In the last century, the search of natural antibiotics, bacteriocins produced by Lactic acid bacteria (LAB) has gained huge attention of scientists establishing an alternative to synthetic antibacterial preservatives and food additives. Since then pure and isolated forms of bacteriocins with a wide range of antimicrobial/antibacterial spectra, reliability and resistivity has lead towards promising tool for natural bio preservatives on the edge of food and pharmaceutical industries [3]. More than 200 bacteriocins from LAB have been isolated and characterized but only half of them were reported at the protein or DNA levels [4]. This identification and screening of bacteriocins were previously followed by instrumental or molecular method with a drawback of time consumption [5]. To overcome this problem, chromatographic, electrophoretic, immunoassays and spectroscopic technologies have been introduced to analyze inhibitory activity and other parameters in case of medicinal plants or species analysis. As the amount of information of bacterial growth and genomic data increases, bioinformatics allow identification at genomic level followed by the reversed-phase High performance liquid chromatography (HPLC) finalizing the purification of the isolated strain [3,5]. For better recoveries certain modifications in maintaining buffer solvents and using different salts for ion exchange chromatography [3].

Characterization of newly isolated bacteriocins mainly done on the basis of measuring the molar mass by SDS-PAGE which provide good resolution of smaller peptides followed by different staining methods [6]. Another reliable method is matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) producing faster results with higher sensitivity than SDS-PAGE characterizing the structural properties as well. However, MALDI-TOF-MS have disadvantage upon detection of cationic adduct clusters (sodium and potassium) in spectra concerning towards decreased sensitivity. Thus, for high throughput Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS) is used to determine the sequence of amino acid chains of bacteriocins [7]. Reenan Van et al., reported thre isolation ,purification and characterization of a novel bacteriocin plantaricin 423 obtained from probiotics isolated from sorghum beer. Plantaricin 423 shows good

antimicrobial activity against bacterial species such as Bacillus cereus, , Enterococcus faecalis, Listeria spp Clostridium sporogenes. and Staphylococcus spp. Plantaricin 423 is found to be active at temperature upto 80 °C but looses half viability when exposed to 100 °C for 60 minutes. Plantaricin 423 tolerates well a pH ranging from 1-10 and is inactivated by enzymes such as pepsin, trypsin, papain and protenase K. Lactobacillus plantarum 423 is allowed to grow in MRS broth and the cells were harvested from centrifugation. Bacteriocin i.e Plantaricin 423 is isolated from the cell free supernatant by the method described by green et al., 1997 [8]. The bacteriocin was further purified by reverse phase FPLC. The molecular weight of plantaricin 423 was found to be 3-5 3·5 kDa as determined by SDS-PAGE. Antimicrobial studies of plantaricin 423 against the active growing cells of O. oeni 19Cl (36 hour incubation) resulted in a slow decrease in viable cell count (2·5×106 to 1×106 cfu ml−1) over a incubation period of initial 24 h, after which a stable state is attained for remaining incubation period [9].

Since lot of studies have been reported on the isolation and characterization of bacteriocins, critically comparing the isolation and screening approaches, in this study we isolate and purify the bacteriocins obtained from probiotic curd isolates and note the effect of temperature, pH and bile salts on antimicrobial activity of bacteriocins.

2. MATERIALS AND METHODS

Indicator strain of microorganism used in this study is ordered from MTCC Chandigarh. The indicator strain included in this study is Propniobacterium acnes. MRS media and other chemicals used were obtained from the research laboratory of Rapture Biotech Noida. Candle jar method is used to provide anaerobic environment for the growth of microorganisms. Bacteria are sub cultured and isolated by streak plate method.

3. METHODOLOGY

3.1 Bacterial Strains, Media and Cultivation Conditions

The media used for the cultivation of microorganisms is the MRS (Man, Rogosa, Sharpe) media . Bacteriocin producing probiotics are isolated from curd culture by streak plate method. Isolates are allowed to grow anaerobically and incubated at 37° C in BOD incubator.

3.2 Isolation, Screening and Identification of Bacteriocin Synthesizing Strains

Homemade yoghurt is used for the production of bacteriocins. About 12 grams of yoghurt sample is allowed to mix in 60 ml of 0.9% w/v of a sterile NaCl solution. The mixture is then vortexed and 8 fold serial dilution's were prepared . 0.1 ml suspension of this sample is plated on De Man, Rogosa, and Sharpe (MRS) agar media by using spread plate technique. The MRS agar plates previously inoculated with the probiotic microorganisms were incubated anaerobically at 37° C using the candle jar method for a maximum of about 48 hrs as shown in Fig. 1. About seven different colonies grow on each plate. These colonies are then sub cultured on the MRS medium for further screening of isolates producing potentially active bacteriocins as shown in Fig. 2. All isolates were recognized and identified, morphologically under a bright field microscope and later on with the phenotypic methods of identification.

3.3 Anaerobic Culturing of Microorganisms

A lighted candle was used as a source of CO_2 generator and 1 % methylene blue strip was used as an indicator of anaerobic environment. An acidic solution of copper sulphate was prepared. Steel wool (5 g) was dipped in freshly prepared acidified copper sulphate solution. The color of steel wool gets converted to dark grey. The steel wool is then spread over an open Petri plate. Thin strips of Whatman filter paper soaked in 1% (w/v) methylene blue solution is used as an indicator. The inoculated plates were kept at the bottom of an air tight glass jar. Then, the Petri dish containing acidic solution of copper sulphate treated steel wool and a candle was kept on top of the inoculated plates. Methylene blue indicator strips were placed inside the jar. Candle was lighted and the mouth of the jar is tightly closed. Maximum proportion of the oxygen present in the jar is consumed by the lightened candle and produced CO_2. The remaining oxygen is consumed by the Iron wool treated with acidified copper sulphate. The jar was then placed in an BOD incubator, to promote the growth of probiotic microorganisms, at 37°C for 48 h [10].

Fig. 1. A) Vortexing of the curd culture mixed with 0.9% sterile saline. B) Anaerobic environment provided to microorganisms by candle jar method. C) Incubation in BOD incubator at a temperature of 37ºC

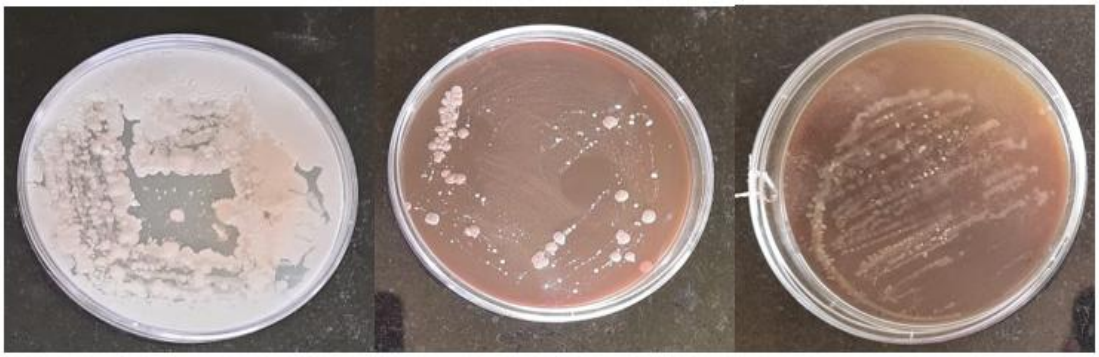


Fig. 2. Subculturing of microorganisms from curd culture

3.4 Detection of Antimicrobial Activity

The bacteriocin producing probiotic isolates were screened for antimicrobial activity by disc-diffusion method [11,12].The test organisms was inoculated to about 50 ml of MRS broth and placed in BOD incubator for 24 hrs at 37° C. After incubation, for about 48 hrs, MRS broth containing the test organisms was centrifuged at 2500 x g for 5 minutes. An MRS plate was prepared by inoculating the indicator microorganism by the spread plate method as shown in Fig. 3. Paper disc is dipped in the supernatant placed on the agar plate inoculated with the test microorganism. The plates were then incubated in BOD incubator at 37° C for 18-24 hrs. The plates were examined for appearance of clear zones of inhibition around the discs impregnated in bacteriocins, which indicate the potency of bacteriocin produced by the test organisms. The size of the zone of inhibition around the discs was measured in millimetres and was recorded.

3.4.1 Extraction of bacteriocins and activity of extracted bacteriocins from probiotic strains against Indicator strains

For the extraction of bacteriocins from the culture broth, the solvent extraction method reported by Westley et al., was used [13]. Isolated colonies showing good antimicrobial effect were inoculated into MRS broth (100 ml) and allowed to incubate at 37° C for 24 hours. To a 500 ml separating funnel, MRS culture broth containing the inoculated microorganism is taken along with equal volume of ethyl acetate. The separating funnel was vigorously shaken for about 10 minutes, and then, the content was kept aside and allowed to settle, making two distinct layers comprising of organic phase of ethyl acetate and aqueous phase. Bacteriocins are present in the upper organic layer and are separated carefully. The solvent was removed and the final extract was allowed to dry and then dissolved in 1 ml of methanol. pH of the supernatant was adjusted to 6 with 1 M NaOH to abolish inhibitory activity

Fig. 3. Preparation of MRS agar media in laminar air flow

from acid followed by addition of 5 mg/ml catalase to get the supernatant free from the antimicrobial effect of hydrogen peroxide. The extract was then subjected to membrane filtration and passed through a 0.20 µm pore size membrane filter and was kept in glass vials.

3.5 Physical and Biochemical Characterization of Bacteriocins

The effects of physiochemical parameters like temperature, pH, bile salts was observed on the stability and activity of purified bacteriocins extracted from Lactobacillus species. These conditions affect the antimicrobial activity of bacteriocins against the indicator strain.

3.5.1 Effect of pH on bacteriocin activity

Bacteriocin (0.5 ml) was added into MRS broth (4.5 ml) at different pH values (3 to 11) and incubated in a BOD incubator for 30 minutes at 37°C. Bacteriocin was the assayed against indicator microorganisms by the disc diffusion method, and activities were compared to nonexposed bacteriocins as a control [14].

3.5.2 Effect of temperature on bacteriocin activity

The extracted bacteriocin was exposed different temperatures for 15 minutes. Then, their antagonistic activities were tested using the disc-diffusion method against the indicator microorganism. Non exposed bacteriocin was taken as a control [15].

3.5.3 Effect of bile salts on bacteriocin activity

To test affect of pH on the bacteriocin activity, 0.5 ml of purified bacteriocin was added into MRS broth (4.5 ml) at different pH values ranging from 3 to 11, and incubated for 30 minutes at 37° C in BOD incubator. Bacteriocin samples were exposed to different pH values and were assayed for antimicrobial activity against indicator organisms especially the gram +ve bacteria by the agar well diffusion method, and activities were compared to nonexposed bacteriocins as a control [15].

4. RESULTS

4.1 Isolation, Screening and Identification of Bacteriocin Synthesizing Strains

Maximum bacteriocin production from lactobacillus species occurred in the log phase of microbial growth. Among seven different isolates, only four isolates showed antimicrobial activity against the indicator strain. The disc- diffusion method 54 used to study the antimicrobial activity of probiotics revealed that four isolates showed the most significant activity against bacteria like P.acnes after 18 - 24 hours of incubation at 37° C .Three of the isolates showed no antimicrobial activity as shown in Fig. 3 and the zone of

inhibition in mm is shown in Table 1. The isolates which shows antimicrobial activity against the indicator strain is further sub cultured for bacteriocin production. The bacteriocin producing isolates were identified using the biochemical or phenotypic tests of identification. The results are shown in Table 2.

Probiotic bacterial isolates were grown in Man, Rogosa and Sharpe (MRS) medium at pH 5.5. All the bacterial isolates were produced small, irregular and round shape colonies, shiny whitish cream or brownish in color and are morphologically similar to bacterial colonies belonging to the *Lactobacillus* family.

The four isolates showing significant antimicrobial activity were examined under bright field microscope to observe their microscopic features. These isolates were found gram positive, short and medium rod-shaped non-spore forming bacterium which indicate them to be member of *Lactobacillus* family. The results are enlisted in Table 1.

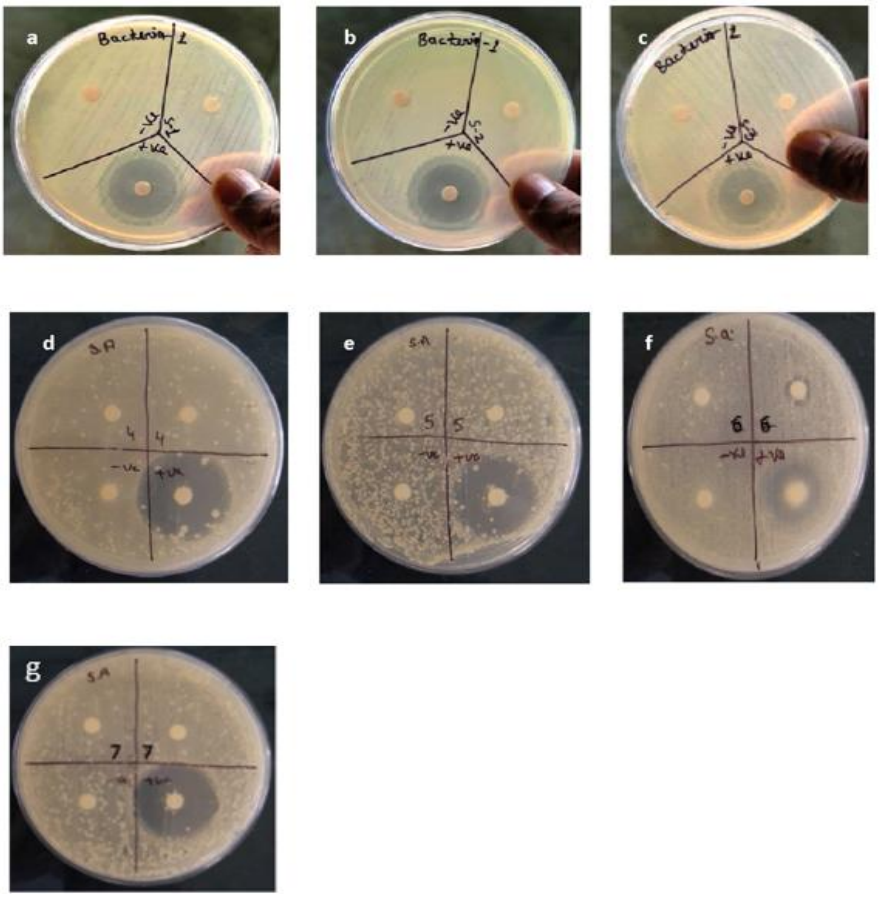


Fig. 4. Zone of inhibition by disc-diffusion method of isolates obtained from curd culture. Figure a, b, c, d, e, f, g are isolates 1, 2, 3, 4, 5, 6 and 7 respectively

Table 1. Zone of inhibition shown by isolates from curd culture

S.No	Positive Control	Negative Control	Zone of Inhibition of Isolates	Interpretation
Isolate 1	30 mm	No inhibition	0 mm	.No antimicrobial activity
Isolate 2	28 mm	No inhibition	0 mm	No antimicrobial activity
Isolate 3	32 mm	No inhibition	0 mm	No antimicrobial activity
Isolate 4	33 mm	No inhibition	2 mm	Hazy activity
Isolate 5	32 mm	No inhibition	10 mm	Clear zone of inhibition
Isolate 6	29 mm	No inhibition	11 mm	Clear zone of inhibition
Isolate 7	30 mm	No inhibition	8 mm	Clear zone of inhibition

Table 2. Phenotypic identification of the different microbial isolates obtained from probiotic curd culture

Identification parameters	Isolate 4	Isolate 5	Isolate 6	Isolate 7
Colony Morphology	Creamish white round colonies	Shiny white round colonies	White irregular colonies	Creamish white irregular colonies
Microscopic view	Rod shaped	Round shaped	Rod shaped	Rod shaped
Gram staining	+	+	+	+
Catalase test	-	-	-	-
Oxidase test	-	+	-	-
Indole test	-	-	-	-
Carbohydrate fermentation test	+	+	+	+
Gas production from glucose	-	-	+	-
Arginine hydrolysis	-	-	-	-
Nitrate reduction	-	-	-	-
Citrate utilization test	-	-	-	-
Acid and bile tolerance	+	+	+	+

4.2 Activity of Extracted Bacteriocin against Indicator Strain, *In-vitro* Study

Bacteriocins obtained from probiotic isolates were purified using the organic solvent extraction method. The bacteriocins are shaken vigorously in a separating funnel containing ethyl acetate and water. It is then kept aside for few minutes. Here, the upper organic phase consisting of ethyl acetate contains bacteriocins, and the lower aqueous layer contains the media constituents and microbes. The antimicrobial activities of bacteriocin extracted from the lactobacillus species present in curd were tested against Propniobacterium acnes which is used as an indicator strain using the disc- diffusion method. A similar pattern for the antimicrobial activity of bacteriocins against microbes responsible for food poisoning was reported by Abo-Amer [16]. The partially purified Bacteriocin obtained from solvent extraction method and isolated from Lactobacillus species showed strong activity against P. acnes.

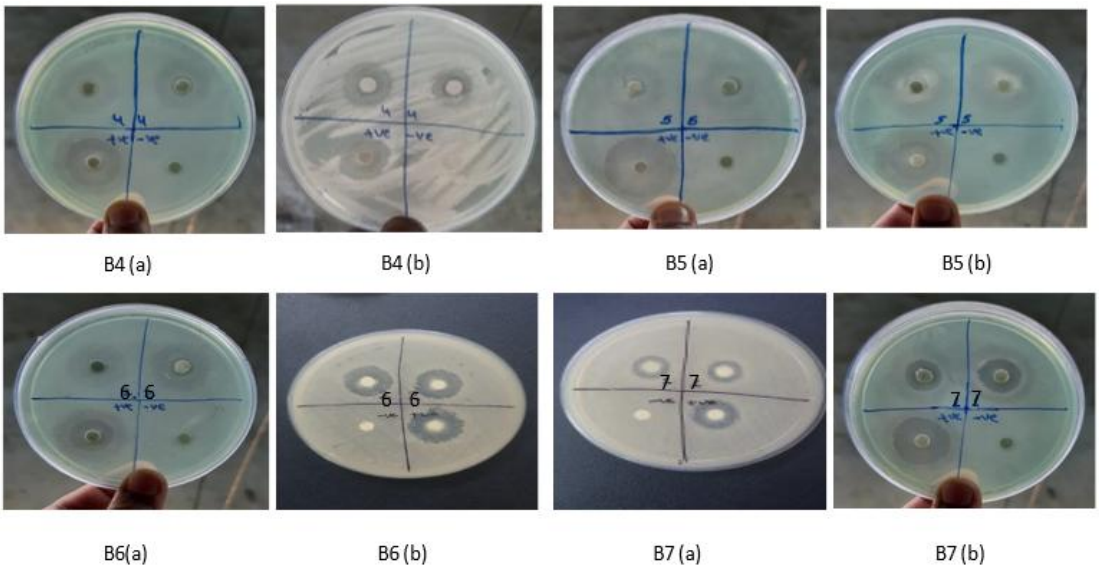


Fig. 5. MRS plates inoculated with indicator strain showing zone of inhibition by different bacteriocins. Two plates are there for each bacteriocin and hence four readings are there each plate contains a positive control a negative control and two readings showing zone of inhibition of bacteriocins

Table 3. Bacteriocin secreted by isolate 6 proves to have maximum antimicrobial activity of 32,34,33 and 32 mm respectivily

Bacteriocins	Indicator strain	Zone of Inhibition in mm						
		Positive control (Clindamycin)		Negative control	Bacteriocin			
B 4	Propniobacterium	35	33	0	30	32	32	28
B 5	acnes	32	34	0	28	29	32	31
B 6		35	32	0	32	34	33	32
B 7		30	32	0	26	28	24	28

4.3 Physical and Biochemical Characterization of Bacteriocin

4.3.1 Effect of pH on bacteriocin activity

Table 4A. Bacteriocins shows maximum efficacy in pH range of 5-6. Efficiency decreases in strong acidic and alkaline pH

Test microorganism	Indicator Strain	Zone of inhibition (mm)vs. pH variations								
		1	2	3	4	5	6	7	8	9
Isolate 4	P.acnes	2.1	12.5	20	25	30.8	22.2	10.3	3.5	0
Isolate 5	P.acnes	1.7	8.5	12	21	29.8	25	6	2.1	0
Isolate 6	P.acnes	1.2	9.1	13.5	25.6	32.5	26	6.5	4	0
Isolate 7	P.acnes	0.25	6.4	10	22	27.6	21	4.3	1.5	0

Maximum antimicrobial effect is observed at a pH range of 3 – 7. Activity completely diminishes at pH 9.

Table 4B. Effect of temperature on bacteriocin activity

Test microorganism	Indicator Strain	Zone of inhibition (mm)vs. Temperature variations								
		30	40	50	60	70	80	90	100	110
Isolate 4	P.acnes	30.5	26.3	22	11.6	6.9	0	0	0	0
Isolate 5	P.acnes	29	24.2	16.4	10.4	6.6	0	0	0	0
Isolate 6	P.acnes	32	25.6	18.9	12.8	3.8	0	0	0	0
Isolate 7	P.acnes	28	22.1	15.4	6.9	4	0	0	0	0

The isolated bacteriocins shows negligible activity at a temperature exceeding 80°C. Maximum antimicrobial activity is observed at a temperature range of 30 – 40°C.

Table 5. Effect of bile salts on bacteriocin activity

Test microorganism	Indicator Strain	Zone of inhibition (mm)vs. Bile salts (solution variations in %)								
		0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
Isolate 4	P.acnes	8	14.6	28	15	2	1.4	0.29	0.02	0.03
Isolate 5	P.acnes	5.8	12.8	27	14	6	2.1	0.21	0	0
Isolate 6	P.acnes	3.9	15.7	30	14.5	8.5	2.3	0.3	0.02	0
Isolate 7	P.acnes	4.2	11.3	22	13.7	4.3	1.8	0.29	0	0

All bacteriocins shows a decrease in antimicrobial activity if bile salt concentration increases beyong 0.5 %. Maximum antagonistic action is seen at a concentration range of 0.2- 0.4%.

Bacteriocins produced from probiotic microorganisms proves to be a better choice of antimicrobials as compared to the synthetic analogues as they are having less side effects and economic as well.

5. DISCUSSION

Bacteriocins are the natural antimicrobial agents produced by some probiotic strains holds promising role in pharmaceutical formulations. They are widely used in a range of topical formulations used to treat dermatological disorders like acne, skin dermatitis etc. Bacteriocins are generally isolated from probiotics present in fermented food products, human fecal matter etc. Nisin, Palantaricin, Natamycin etc are some of the widely used bacteriocins used as preservatives in food industry. The probiotics used for bacteriocin production are categorized as GRAS (Generally Recognized as Safe). Bacteriocins fused with metallic nanoparticles like gold, silver and zinc nanoparticles have increased antimicrobial activity against the pathogenic strains. Bacteriocins can tolerate high temperature even up to 70°C and active in pH range of 3-9.

6. CONCLUSION

Bacteriocins produced from probiotics isolated from curd shows good antimicrobial activity against the acne causing bacteria i.e Propniobacterium acnes and hence widely used in antiacne formulations including topical formulations like creams, gels, serum and ointments. Probiotics proves to be a better choice of drug in future rather than other antimicrobials in the present. Bacteriocins encapsulated with zinc and silver have enhanced antimicrobial activity.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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