

Original Research Article

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

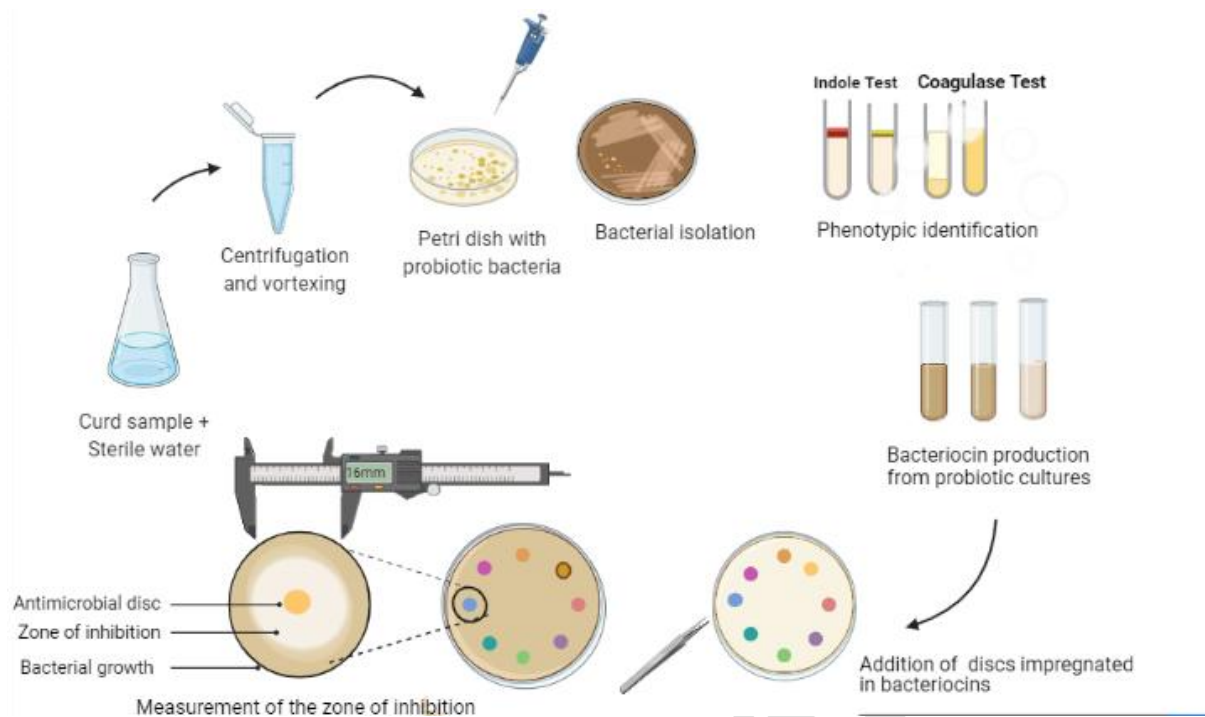
ABSTRACT:

Introduction: Health benefits offered by probiotics and use of probiotics in wide range of clinical disorders is well established and scientifically substantiated. 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, *Propionibacterium acnes*. Bacteriocins are isolated, purified and dried. Morphological and general phenotypic tests are performed for identification of microorganisms like carbohydrate fermentation test, catalase test, indole test and coagulase test. 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.

Materials & Methods: *P.acnes* (indicator strain) is procured from MTCC Chandigarh. All chemicals are obtained and antimicrobial studies are performed at Rapture Biotech, Noida. Streak plate method is used for isolation of microorganisms.

Result: 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.

Graphical Abstract



Keywords: Bacteriocins, Probiotics, Identification, Purification, Antimicrobial, Zone of inhibition, Streaking, Disc- diffusion.

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 proteinaceous 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 biopreservatives 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].

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 *Propionibacterium 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 subcultured and isolated by streak plate method.

3. METHODOLOGY

3.1 Bacterial strains, Media and Cultivation conditions

MRS (Man, Rogosa, Sharpe) media is used for the cultivation of microorganisms. 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 normal saline and vortexed. This mixture is then serially diluted eight times. 0.1 ml suspension of this sample is plated on De Man, Rogosa, and Sharpe (MRS) agar media by the spread plate method. The MRS agar plates were incubated anaerobically at 37° C using the candle jar method for a maximum of about 48 hrs. About seven different colonies grow on each plate. These colonies are then subcultured on the MRS medium for further screening of bacteriocin-producing isolates. All isolates were identified by the biochemical method of identification.



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 37° C.*



Fig. 2: *Subculturing of microorganisms from curd culture*

2.3 Anaerobic culturing of microorganisms.

A lighted candle was used as a source of CO₂ generator and one per cent methylene blue strip was used as an indicator of anaerobiosis. Acidified 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 one per cent (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 open plate containing acidified copper sulphate treated steel wool and a candle was kept on top of the inoculated plates. Methylene blue indicator strips were kept 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 CO₂. The remaining oxygen is consumed by the Iron wool treated with acidified copper sulphate. The jar was then placed in an BOD incubator at 37°C for 48 h [8].

2.4 Detection of antagonistic activity

The bacteriocin producing isolates were screened for antimicrobial activity by the disc- diffusion method [9]. 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, broth containing the test organisms was centrifuged at 2500 x g for 5 minutes. Following incubation, An MRS agar plate was prepared by inoculating the indicator microorganism by the spread plate method. Paper disc is dipped in the supernatant placed on the agar plate inoculated with the test microorganism. The plates were then incubated at 37° C for 18-24 hrs. The plates were examined for clear zones of inhibition around the discs impregnated in bacteriocins, which indicate the bacteriocin activity of the test organisms. The size of the zone of inhibition was measured in millimetres and was recorded.



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

2.4.1 *Extraction of Bacteriocins and Activity of extracted bacteriocins against Indicator strains.*

For the extraction of bacteriocins the solvent extraction method reported by Westley et al. was used. from the selected strains [10]. Isolated colonies showing good antimicrobial effect were inoculated into MRS broth (100 ml) and incubated at 37° C for 24 hours. To a 500 ml separating funnel, 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 allowed to settle, making two distinct layers of organic phase and aqueous phase. Bacteriocins are present in the upper organic layer and are separated carefully. The solvent was removed and the final dried extract was dissolved in 1 ml of methanol. pH of the supernatant was adjusted to 6 with 1 M NaOH to eliminate inhibitory activity 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 stored in glass vials.

2.5 Physical and Biochemical characterization of bacteriocins.

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

2.5.1 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.

2.5.2 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.

2.5.3 Effect of bile salts on Bacteriocin activity.

To test affect of pH on the bacteriocin activity, bacteriocin extract (0.5 ml) was added into MRS broth (4.5 ml) at different pH values (3 to 11) and incubated for 30 minutes at 37° C. Bacteriocin samples were exposed to different pH values and were assayed against indicator organisms by the agar well diffusion method, and activities were compared to nonexposed bacteriocins as a control.

3. RESULTS AND DISCUSSION

3.1 Isolation, Screening and Identification of Bacteriocin synthesizing strains

Maximum bacteriocin production from *Lactobacillus* species occurred in the log phase of growth. Among seven different isolates, only four isolates showed antimicrobial activity against the indicator strain. The disc- diffusion method revealed that four isolates showed the most significant activity against *P.acnes* after 18 - 24 hours of incubation at 37° C .Three of the isolates showed no antimicrobial activity. 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.

Isolates were grown in Man, Rogosa and Sharpe (MRS) medium at pH 5.5. All the isolates were produced small, irregular and round shape with shiny whitish cream or brownish colored which were morphologically similar to *Lactobacillus* species

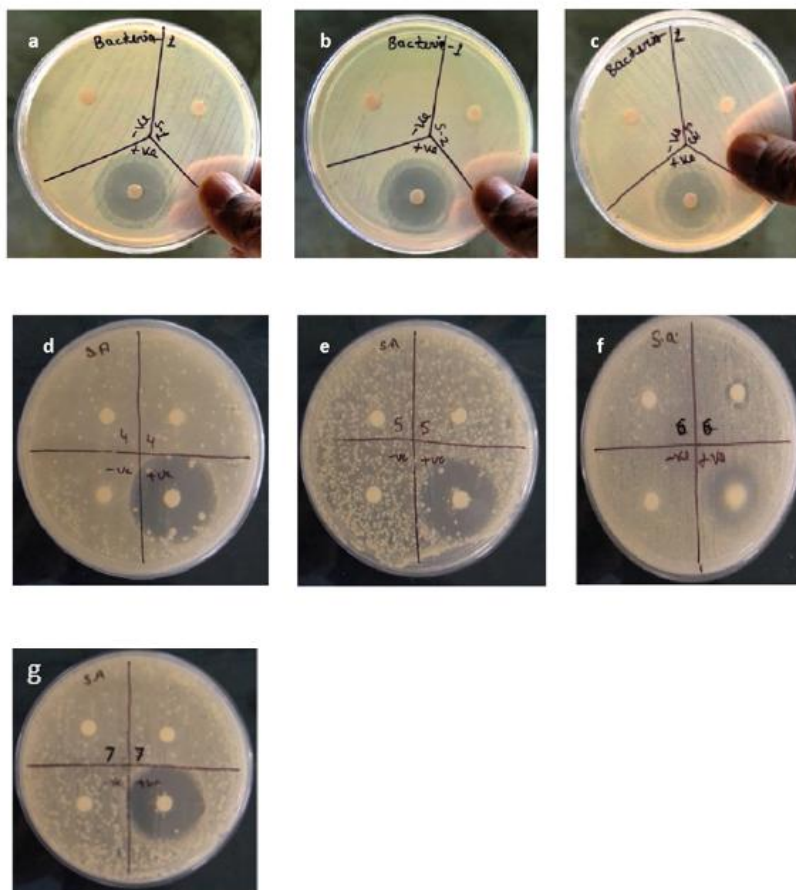


Fig: 4 Zone of inhibition by disc- diffusion method of isolates obtained from curd culture. Figure a,b,c,d,e,f,g are isolates 1,2,3,4,5,6,and 7 respectively.

<u>S.No</u>	Positive Control	Negative Control	Zone of Inhibition of Isolates	Interpretation
Isolate 1	30 mm	No inhibition	0 mm	<u>No</u> antimicrobial activity
Isolate 2	28 mm	No inhibition	0 mm	Hazy activity
Isolate 3	32 mm	No inhibition	0 mm	No antimicrobial activity
Isolate 4	33 mm	No inhibition	2 mm	No antimicrobial 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 1: Zone of inhibition shown by isolates from curd culture.

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.

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	+	+	+	+

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

3.2 Activity of extracted bacteriocin against Indicator strain, in-vitro study.

Bacteriocins obtained from *Lactobacillus* sp. isolates was purified using the organic solvent extraction method. 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 *Propionibacterium 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 [11]. The partially purified Bacteriocin isolated from *Lactobacillus* species showed strong activity against *P.acnes*.

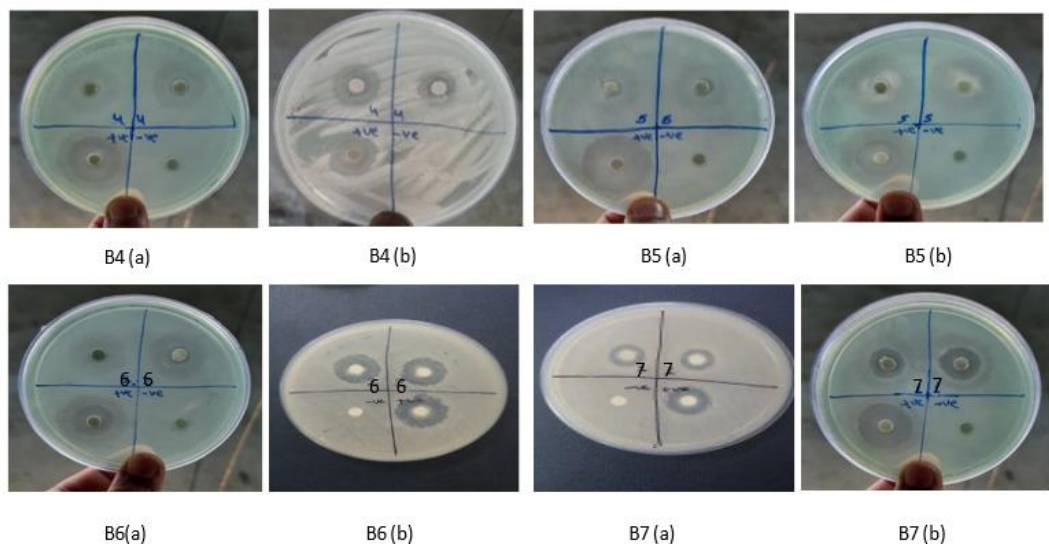


Fig:5 MRS plates inoculated with indicator strain showing zone of inhibition by different bacteriocins. Two plates are there for each bacteriocin and hence four readings are there Each plate contains a positive control, a negative control and two readings showing zone of inhibition of bacteriocins.

Bacteriocins	Indicator strain	Zone of Inhibition in mm						
		Positive control (Clindamycin)		Negative control	Bacteriocin			
B 4	Propniobacterium acnes	35	33	0	30	32	32	28
B 5		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

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

3.3 Physical and Biochemical characterization of bacteriocin

3.3.1. Effect of pH on bacteriocin activity

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	<u>P.acnes</u>	2.1	12.5	20	25	30.8	22.2	10.3	3.5	0
Isolate 5	<u>P.acnes</u>	1.7	8.5	12	21	29.8	25	6	2.1	0
Isolate 6	<u>P.acnes</u>	1.2	9.1	13.5	25.6	32.5	26	6.5	4	0
Isolate 7	<u>P.acnes</u>	0.25	6.4	10	22	27.6	21	4.3	1.5	0

Table 4: Effect of pH on bacteriocin activity

3.3.2. 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	<u>P.acnes</u>	30.5	26.3	22	11.6	6.9	0	0	0	0
Isolate 5	<u>P.acnes</u>	29	24.2	16.4	10.4	6.6	0	0	0	0
Isolate 6	<u>P.acnes</u>	32	25.6	18.9	12.8	3.8	0	0	0	0
Isolate 7	<u>P.acnes</u>	28	22.1	15.4	6.9	4	0	0	0	0

Table 5: Effect of temperature on bacteriocin activity

3.3.3. Effect of bile salts on Bacteriocin activity

Test microorganism	Indicator Strain	Zone of inhibition (mm)vs. Bile salts (solution variations in %								
		3	4	5	6	7	8	9	10	11
Isolate 4	<u>P.acnes</u>	8	14.6	28	15	2	1.4	0.29	0.02	0.03
Isolate 5	<u>P.acnes</u>	5.8	12.8	27	14	6	2.1	0.21	0	0
Isolate 6	<u>P.acnes</u>	3.9	15.7	30	14.5	8.5	2.3	0.3	0.02	0
Isolate 7	<u>P.acnes</u>	4.2	11.3	22	13.7	4.3	1.8	0.29	0	0

Table 6: Effect of bile salts on Bacteriocin activity

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.

CONCLUSION:

Bacteriocins produced from probiotics isolated from curd shows good antimicrobial activity against the acne causing bacteria i.e Propionibacterium 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.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. 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.

REFERENCES:

- [1]. Baquero, F., & Moreno, F. (1984). The microcins. *FEMS microbiology letters*, 23(2-3), 117-124.
- [2]Yang, S. C., Lin, C. H., Sung, C. T., & Fang, J. Y. (2014). Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. *Front. Microbiol.*, 5, 241.
- [3]. Kaškonienė, V., Stankevičius, M., Bimbiraitė-Survilienė, K., Naujokaitytė, G., Šernienė, L., Mulkytė, K., ... & Maruška, A. (2017). Current state of purification, isolation and analysis of bacteriocins produced by lactic acid bacteria. *Appl. Microbiol. Biotechnol.*, 101(4), 1323-1335.
- [4]. Alvarez-Sieiro, P., Montalbán-López, M., Mu, D., & Kuipers, O. P. (2016). Bacteriocins of lactic acid bacteria: extending the family. *Appl. Microbiol. Biotechnol.*, 100(7), 2939-2951.
- [5]. Chumchalova, J., Stiles, J., Josephsen, J., & Plockova, M. (2004). Characterization and purification of acidocin CH5, a bacteriocin produced by *Lactobacillus acidophilus* CH5. *J. Appl. Microbiol.*, 96(5), 1082-1089.
- [6]. Gao, Y., Li, D., Liu, S., & Zhang, L. (2015). Garviecin LG34, a novel bacteriocin produced by *Lactococcus garvieae* isolated from traditional Chinese fermented cucumber. *Food Control*, 50, 896-900.
- [7]. Zou, J., Jiang, H., Cheng, H., Fang, J., & Huang, G. (2018). Strategies for screening, purification and characterization of bacteriocins. *Int. J. of Biol. macromol.*, 117, 781-789.

- [8]. Saha, U. S., Misra, R., Tiwari, D., & Prasad, K. N. (2016). A cost-effective anaerobic culture method & its comparison with a standard method. *The Indian J. Med. Re.*, 144(4), 611.
- [9]. Tagg, J., & McGiven, A. (1971). Assay system for bacteriocins. *J. Appl. Microbiol.*, 21(5), 943-943.
- [10]. Westley, J. W., Evans Jr, R. H., Sello, L. H., & Troupe, N. (1979). Isolation and characterization of antibiotic x-14547a, a novel monocarboxylic acid ionophore produced by streptomyces antibioticus nrrl 8167. *The Journal of antibiotics*, 32(2), 100-107.
- [11]. Abo-Amer, A. E. (2007). Characterization of a bacteriocin-like inhibitory substance produced by *Lactobacillus plantarum* isolated from Egyptian home-made yogurt. *Sci. Asia*, 33, 313-319.

UNDER PEER REVIEW