Vol.64: e21190749, 2021
https://doi.org/10.1590/1678-4324-2021190749
ISSN 1678-4324 Online Edition

Article - Agriculture, Agribusiness and Biotechnology

Production, Purification and Characterization of a Novel Bacteriocin Produced by *Bacillus subtilis* VS Isolated from Mango (*Mangifera indica* L.)

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Editor-in-Chief: Paulo Vitor Farago
Associate Editor: Acácio Antonio Ferreira Zielinski

Received: 2019.12.11; Accepted: 2020.12.18.

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HIGHLIGHT

- Bacillus subtilis VS, an isolate from mango with bacteriocin producing ability.
- Bacteriocin potentially inhibited pathogenic S. boydii, S. typhi & amp; L. monocytogenes.
- DEAE cellulose and sephadex G75 chromatography are best suited for purification.
- Purified bacteriocin found to be stable at broad pH and temperature range.

Abstract: Bacteriocin has been identified as an excellent alternative to chemical preservatives due to its astonishing antimicrobial activity against food spoiling and food-borne pathogens. So there is a need to identify the newer and potent sources of bacteriocin producers. This study aims the isolation of potent bacteriocin producing microorganism from fresh fruits and vegetables, its production, purification, and characterization.

Firstly, 43 isolates were analysed for its antimicrobial potential, out of which7 were found to inhibit the growth of various pathogens. Considering the results of antimicrobial activity; the microorganism isolated from mango was regarded as the most potent one; which was identified as Bacillus subtilis VS.70% ammonium sulphate precipitated and dialysed bacteriocin was purified using DEAE cellulose and sephadex G75 chromatography. Bacteriocin was purified by 24.64 fold with 8.65% recovery and its molecular weight was found to be 31.2kDa. The Purified bacteriocin was found to be stable at broad pH and temperature. It was found to be degraded by various proteases studied confirming its proteinaceous nature. Considering all these attributes; the purified bacteriocin isolated from Bacillus subtilis VS can be exploited by various food industries.

Keywords: Antimicrobial activity; Bacteriocin; Chromatography; Proteases.

INTRODUCTION

The demand for healthier and ready-to-eat products is increasing with ever-growing population. The use of chemicals in day today life has tremendously increased in last few decades in various forms such as fertilizers, pesticides, herbicides and even in food processing industries. By 2030, global food demand is estimatedto accelerate by 35% [1]. This increasing demand can only be fulfilled either by enhancing the food production or by reducing the food waste. Microorganisms are vital part of our ecosystem and playing role in metabolism of human being besides managing nutrient balance on earth. They also produce certain antimicrobial compounds which help in managing microbial consortium. Among these, bacteriocins are ribosomally synthesized extracellularly released bioactive peptides with antagonistic activity which are non-toxic to eukaryotic cells [2]. *Lactococcus, Lactobacillus, Carnobacterium, Enterococcus* and *Streptococcus* are known genera for bacteriocin production possessing a strong antimicrobial activity against food spoiling and human pathogenic microorganisms [3].

They are classified into four different groups based on their primary structure, heat reliable, molecular mass and organization [4]. They have varying inhibitory spectrum which can also be used along with hurdle technology and these characteristics allow their applications in food safety and spoilage[5]. Considering end users interest in natural products and the high cost of food borne illness, packaged food manufacturers are always looking for new ways to preserve the food. This further strengthens the use of bacteriocins which are in high demand in food industry as alternate to chemical preservatives. Bacteriocins are proteinaceous metabolites produced by a large number of bacterial species including Gram positive as well as Gram negative bacteria [6]. Among the bacteriocins of Gram positive bacteria, lactic acid bacteria (LAB) have been dominated the literature due to their GRAS status and ability to be used in food preservation. Till date around 230 LAB bacteriocins have been discovered [7] but only three have been commercialized i.e. Nisin (produced by *Lactococcus lactis*) commercially available as Nisaplin®, pediocin (produced by *Pediococcus acidilactici*) available as Alta 2431® and Micocin® (produced by *Carnobacterium maltaromaticum* UAL307) [8]. Among all the bacteriocins, nisinhas been used in around 48 countries all over the world and aids great social and economic value [9]. The genus *Bacillus* is second most studied genera for bacteriocin production e.g. *Bacillus subtilis* and *Bacillus licheniformis*, these are generally regarded as safe bacteria [10]. This genus includes a number of bacterial species which are industrially essential and exhibits antiquity of secure use for both food and other industries [11]. From last many decades, *Bacillus* spp. are well-known for production of wide variety of peptides/protein and antibiotics.

Considering these facts, the current study was planned with the aim to isolate a potent bacteriocin producer followed by its purification and complete characterization. Bacteriocin producing microorganisms are also found to be reported from natural habitat like fruits and vegetables. Organisms like *Pediococcus pentosaceus* K23-2, *Lactobacillus paracasei* HD1.7, *Lactobacillus plantarum* 24, *Lactobacillus plantarum* ST16Pa, *Bacillus subtilis*, *Leuconostoc pseudomesenteroide 607* are previously isolated from kimchi, Chinese cabbage sauerkraut, marula fruit, papaya, baobab seed (maari) and persimmon fruit, respectively [12-17]. In this study, various fruits and vegetables like mango, banana, lemon, onion, tomato, chilli and carrot were exploredin order to isolate a novel and potent bacteriocin producer.

MATERIALS AND METHODS

Sample Collection

Different fresh and mature food samples of fruit and vegetables (i.e. mango, banana, lemon, onion, tomato, chilli and carrot) were collected in the month of April from relevant trading centers and were stored at 4°C until processed. Samples were identified and confirmed with the help of Botany Department, Amity University Rajasthan.

Isolation of bacteriocin producing strain

25g of each collected sample was weighed (exocarp, mesocarp and endocarp of all the samples except mango in which endocarp was not taken for isolation) and homogenized in 225ml quarter strength ringer solution for 5 minutes. Serially diluted samples were spread plated on tryptic soy agar with 1% yeast extract and incubated at 37°C for 48 hours. After incubation all different type of colonies were isolated and screened for bacteriocin production against indicator organisms [18].

Indicator Bacterial Cultures

Escherichia coli (MTCC 1687), Salmonella typhi (MTCC 3224), Shigellaboydii (MTCC 11947), Staphylococcus aureus (MTCC 737), Pseudomonas aeruginosa (MTCC 1688), Klebsiella pneumonia (MTCC 7407),Listeria monocytogenes (MTCC 657), and Vibrio parahaemolyticus (MTCC 451) were purchased from Microbial Type Culture Collection (MTCC) Chandigarh, and were used as an indicator microorganisms to study the antimicrobial activity of bacteriocins produced by bacterial isolates.

Antimicrobial activity assay

Bacterial isolates were grown in 50mL tryptic soy broth with 1% yeast extract and then centrifuged at 10,000 rpm for 15 minute at 4°C. The supernatant was filtered by 0.22µm size membrane (Millipore, India) and pH of supernatant was adjusted to 6.5. 100µL of supernatant was added into each well on agar plates spreaded with an indicator strain and incubated at 37°C for 24hrs. After incubation the zone of inhibition was measured [19].

Identification and Phylogenetic analysis

The most potent isolate which confirmed the presence of bacteriocin as well as having highest antimicrobial activity was identified by 16S rDNA sequencing. DNA was extracted and amplified by using polymerase chain reaction with 16S rDNA specific primers 8F (5'AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' CGGTGTGTACAAGACCC 3') using Veriti ® 96 well thermal cycler (Model No. 9902). The PCR reactions were performed as: denaturation at 95°C for 1 min, 55°C for 1 min, 30 cycles of 72°C for 1 min and final extension step at 72°C for 10 min. The PCR amplicon was purified and further subjected to Sanger sequencing. The 16S rDNA sequence was analysed by using BLAST tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Based on maximum identity score first fifteen sequences were aligned using multiple alignment software program Clustal W. The homologous sequences of other species were used for phylogenetic analysis and the phylogenetic tree was constructed using MEGA 6 [20].

Effect of incubation period on bacteriocin production

The most potent isolate was inoculated with 1% v/v in the production medium of 100 ml tryptic soy broth supplemented with 1% yeast extract and it was incubated at 37°C and 120 rpm. The production of bacteriocin was monitored upto 48 hours, where growth was measured at 600nm [21] and antimicrobial activity of crude bacteriocin was calculated in terms of AU/ml. The smallest detectable zone of inhibition corresponding to the diluted bacteriocin was used to calculate AU/mL [22].

Production and purification of bacteriocin

The production media of tryptic soy broth with 1% yeast extract was inoculated with most potent isolate at 1% v/v and incubated at 37°C for 36 hours. After incubation whole broth was centrifuged at 10,000 rpm for 15 minutes at 4°C and cell free supernatant was used as crude bacteriocin. The pH of cell free supernatant was adjusted to 6.5 with 1 M NaOH and subjected to determination of antimicrobial activity and protein content. For partial purification of bacteriocin, crude supernatant was precipitated at 70% ammonium sulphate precipitation and held overnight at 4°C. The sample was then centrifuged at 10,000 rpm for 30 min at 4°C and the precipitate was collected and dialysed. The precipitate was further purified with DEAE-cellulose column chromatography (12 × 1 cm) by using an increasing gradient of NaCl (0.1–0.5 M) which was pre-equilibrated with phosphate buffer (50 mM, pH 7.0). Fractions showing the effective antimicrobial activity were collected and final purification was done by Sephadex G-75 column (22 × 1 cm), and the active fraction was collected and used for further studies.Protein concentration was determined by Lowry [23]. The molecular weight of purified protein was estimated by SDS-PAGE analysis.

Biochemical characterization of purified bacteriocin

The purified bacteriocinwas incubated at30, 40, 50, 60, 70, 100 and 121°C for 20 minutes to determine its thermal sensitivity where activity at 30°C was taken as 100%. The pH stability of bacteriocin was determined by adjusting the pH ranges from 3 to 9 by using 1N NaOH or HCl for 20 minutes [24]. The residual activity (AU/ml) was measured at each pH and activity at pH 6.5 was taken as 100%. The enzymatic sensitivity was evaluated by incubating the bacteriocin for 2 hours at 37°C in the presence of 1 mg/ml final concentration of protease, trypsin and pepsin. All the mixtures were heated to 100°C for 5 minutes to inactivate the enzymes [25]. The susceptibility of antimicrobial activity to enzymes was determined by agar well diffusion method.

Statistical analysis

All the above experiments were performed in three replicates, the mean and standard deviation data were expressed. Microsoft Office data analysis tool pack was used to carry out one way ANOVA [26].

RESULTS AND DISCUSSION

Sample processing for isolation of bacteriocin producing strain

Bacteriocins are the bioactive peptides produced by one strain having antagonistic activity against their closely related strains. Bacteriocin producing microorganisms are also found to be reportedin the natural habitat like fruits and vegetables. So, to isolate potent bacteriocin producing microorganism variety of fruits as well as vegetables samples i.e. mango, banana, lemon, onion, tomato, chilli and carrot were screened on tryptic soy agar with 1% yeast extract. After incubation at 37°C for 48 hours, all different types of colonies grown were picked and tested for its bacteriocin producing ability against various indicator strains.

Bacteriocin production and its antimicrobial potential

Forty-three microorganisms were isolated on tryptic soy agar plates supplemented with 1% yeast extract. These isolates were further grown in tryptic soy broth supplemented with 1% yeast extract which then subjected to determine its bacteriocin producing ability against indicator strains (data not shown). Seven potent isolates were selected on the basis of inhibitory effects against most of the indicator strains as well as considering their zone of inhibition (Table 1). Organism (VS10) isolated from the sample of mango at 10^{-5} dilution showed the highest zone of inhibition (Figure 1) against *Shigella boydii* (19.5 mm) followed by *Salmonella typhi*(13 mm), *Listeria monocytogenes* (11 mm), *Pseudomonas aeruginosa* (5 mm), *Escherichia coli* (4.3 mm) and *Staphylococcus aureus* (3.8 mm) whereas; it was inactive against *Klebsiella pneumonia* and *Vibrio parahaemolyticus*.

Table 1: Zone of inhibition (in mm)* of seven potent bacteriocin producing isolates against indicator organisms.

Sr. No.	Strain	*Salmonella typhi*	*Shigellaboydii*	*Listeria monocy-togenes*	*Pseudom-onas aeruginosa*	*Staphyloco-ccus aureus*	*Escherichia coli*	*Klebsiellapneumon-iae*	*Vibrio parahaemo-lyticus*
1	VS08	6.4±0.04	3.2±0.10	5.7±0.20	-	6.8±0.47	7.0±0.30	3.8±0.22	-
2	VS10	13±0.25	19.5±1.16	11±0.46	5±0.25	3.8±0.31	4.3±0.13	-	-
3	VS13	14±0.31	9.0±0.57	12±0.80	-	6.8±0.26	4.8±0.15	-	3.7±0.21
4	VS24	8.1±0.06	7.2±0.25	4.7±0.20	2.6±0.11	3.2±0.23	4.3±0.10	-	5.4±0.32
5	VS28	7.8±0.23	6.4±0.26	6.4±0.15	-	4.8±0.25	3.6±0.14	2.7±0.14	-
6	VS34	5.3±0.10	3.1±0.35	4.5±0.44	-	2.7±0.13	6.6±0.20	-	3.4±0.12
7	VS41	6.7±0.07	4.1±0.32	6.3±0.25	2.4±0.15	3.8±0.28	5.6±0.14	-	-

*Values are mean± SD (standard deviation) of three replications.

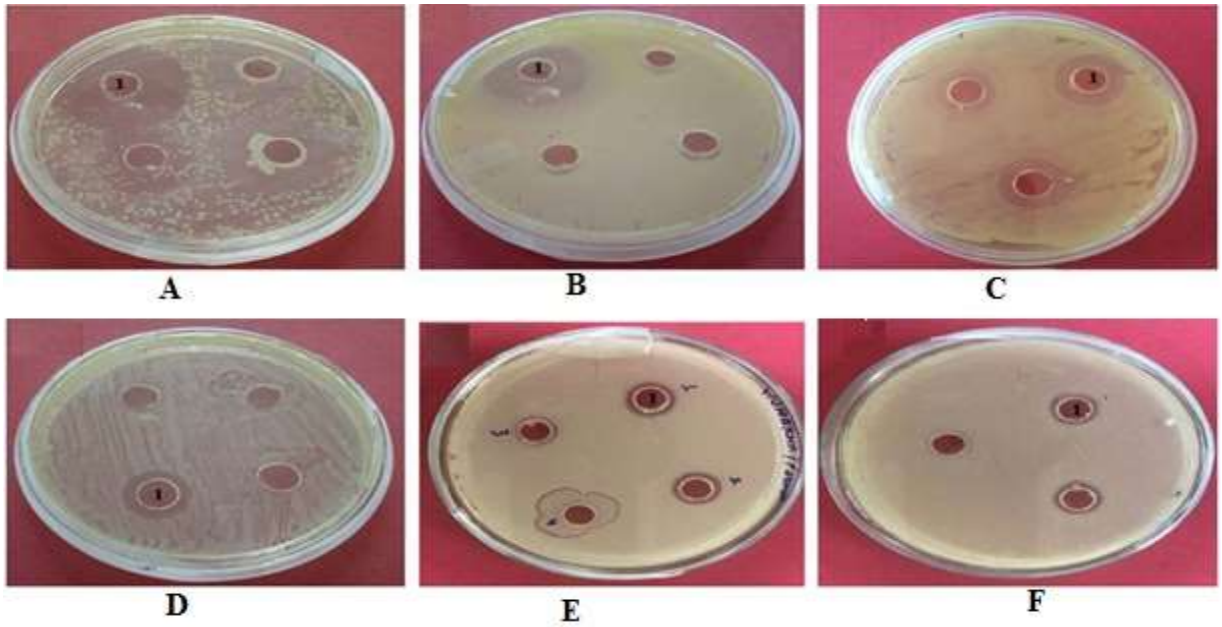

Figure 1. Zone of inhibition of bacteriocin (100 µl in each well) under study (1) against (A) Shigellaboydii (MTCC 11947) (B) Salmonella typhi (MTCC 3224) (C) Listeria monocytogenes (MTCC 657) (D) Pseudomonas aeruginosa (MTCC 1688)(E) Escherichia coli (MTCC 1687) (F) Staphylococcus aureus (MTCC 737)

A study claimed that Gram negative bacteria are not much affected by bacteriocin due to presence of outer membrane [27], in contradiction to which, bacteriocin under this study showed highest activity against both Gram positive and Gram negative bacteria. Similar results were obtained in case of bacteriocin produced by *Enterococci* which was found active against Gram negative bacteria i.e. *Salmonella pullorum* and *Escherichia coli* [28]. Whereas *Lactobacillus plantarum* based bacteriocin also showed strong antimicrobial activity against both Gram positive (*Staphylococcus aureus, Enterococcus fecalis, Listeria monocytogenes*) as well as Gram negative bacteria (*Escherichia coli*) [29]. BU, a broad spectrumbacteriocin produced from *Lactococcuslactis subsp. Lactis*bv. Diacetylactis BGBU1-4 was also found active against both Gram positive and negative bacteria [30].

Identification by 16SrDNA sequencing and phylogenetic analysis

Potent bacteriocin producer isolated in this study was identified by a robust and widely used ribotyping method of 16S rDNA sequencing. A single discrete PCR amplicon band of 1500 bpwas observed (data not shown). The forward and reverse primer data sequence created the consensus sequence of 1372 bp. resulting 16S rDNA sequence was compared with GenBank database using standard nucleotide BLAST ($blast_n$) optimised for highly similar sequences. Obtained result was submitted to GenBank (Accession No. MG493260) and the strain were identified as *Bacillus subtilis* VS. The phylogenetic tree was generated as per the earlier report [26]. The ideal tree with the total of branch length of 48.15 was obtained and it showed evolutionary relationship between *Bacillus subtilis* VS and the other closely related strains (Figure 2). MEGA6 software was employed to carry out the evolutionary analysis.

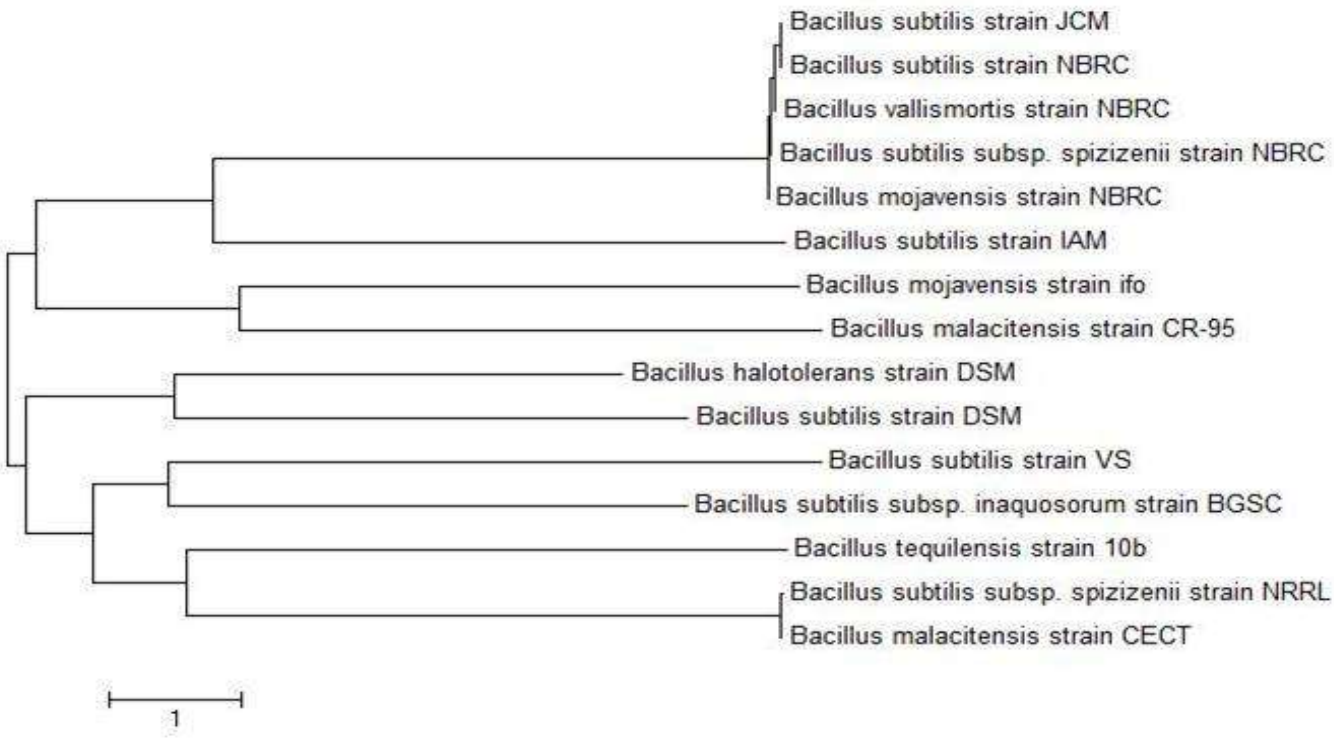

Figure 2. Phylogenetic tree of Bacillus subtilis VS and other closely related Bacillus species

Effect of incubation period on bacteriocin production

The highest production of bacteriocin was found to be at 36 hours of incubation period in tryptic soy broth supplemented with 1% yeast extract, whereas the production was decreased afterwards (Figure3.) Bacteriocin production is generally a growth associated process [31], it enhances simultaneously with cell growth. A bacteriocin Bt BRC-ZYR2 produced from *Bacillus thuringiensis* showed maximum activity during

late log phase[8].The bacteriocin production occurs during the exponential phase reaching its maximum during the stationary phase of the growth curve. Similar phenomenon has been observed in earlier study carried out by Gaspar and coauthors [32].A bacteriocin produced from *Staphylococcus macedonicus*ADC-DC198 isolated from Greek Kasseri cheese showed activity in early exponential phase and reached highest in stationary phase[33]. This similar trend for bacteriocin production has been reported for pediocin, nisin and many other bacteriocins [34].

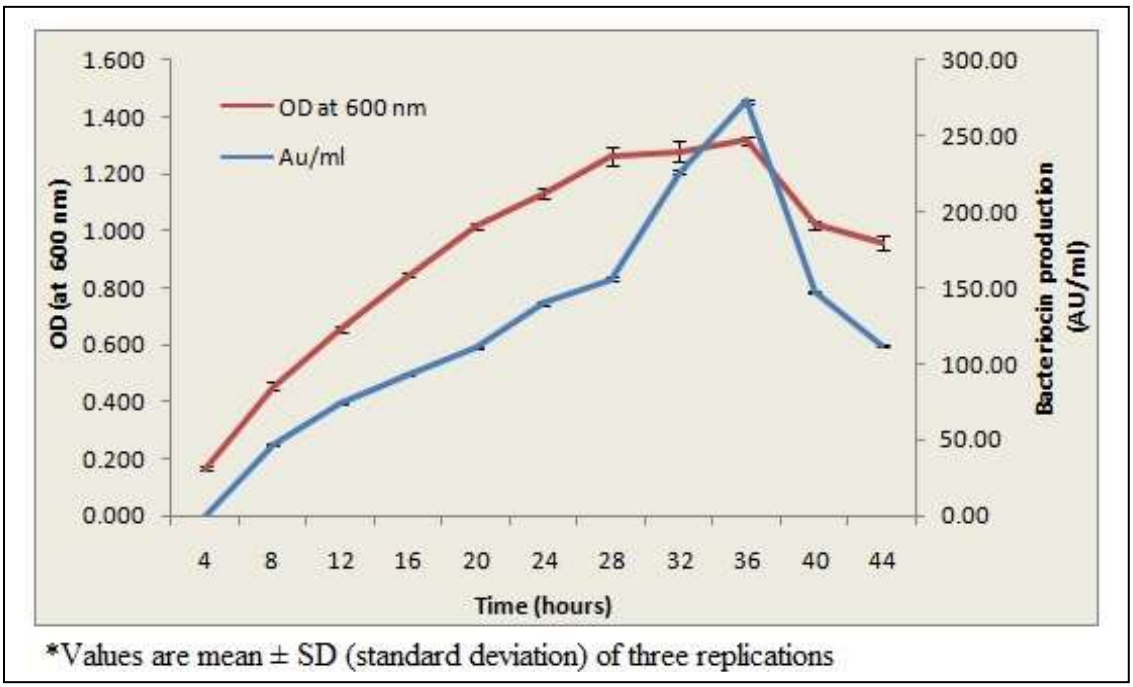

Figure 3. Effect of incubation period on bacteriocin production from Bacillus subtilis VS*.

Purification of bacteriocin

After confirmation of the presence of bacteriocin,itwas purified to the homogenecity. The purification details are summarized in Table 2. Highest precipitation was found to be at 70% of ammonium sulphate saturation which is then subjected to further purification.

Table 2. Summary of purification profile for bacteriocin from *Bacillus subtilis* VS

Purification Stage	Total Activity (AU)	Total Protein (mg)	Specific Activity (AU/mg)	Purification fold	% Recovery
Culture supernatant	27760	319	87.02	1	100.0
Ammonium sulphate precipitation	8520	35.2	242.04	2.78	30.69
DEAE Cellulose	3919.2	4.8	816.5	9.38	14.11
Sephadex G-75	2401.5	1.12	2144.19	24.64	8.65

*Values are mean of three replicas

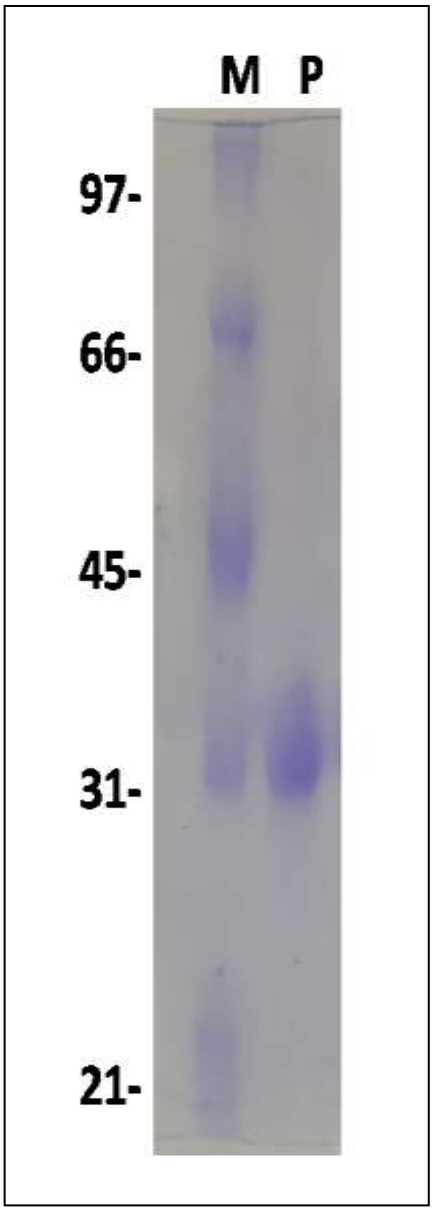

Figure 4. SDS-PAGE of the purified bacteriocin from Bacillus subtilis VS. (M) Protein markers. (P) Purified bacteriocin.

It was found that in every step from crude to final active fraction, the protein content was decreased but antimicrobial activity was gradually increased. The specific activity has increased from 87.02AU/mg to 2144.19 AU/mg upto final active fraction with purification fold 24.64 and yield of 8.65%. Homogenecity of the pure bacteriocin was confirmed by a single band of 31.2kDa obtained in SDS-PAGE as shown in figure 4. Bacteriocin in this study found to be precipitated at 70% ammonium sulphate, generally recovery of most of the bacteriocins found in the range of 60 – 80 % saturation. Similar results were observed in case of Paracin 1.7, bacteriocin from *Lactobacillus paracasei*HD1-7 which showed maximum inhibition zone at 70% level of saturation [24]. However, bacteriocin produced from *Lactococcus lactis* sub sp. *Lactis* strain 63 was partially purified by ammonium sulphate upto 60% saturation only [35]. In this study, further purification of bacteriocin was carried out by using DEAE cellulose column chromatography and sephadex G-75. The activity of purified bacteriocin was increased upto 2144.19 AU/mg with final recovery of 8.65%.Similar results were observed in a study by Boubezari and coauthors [36] where purified bacteriocin from strain *Escherichia coli* P2C isolated from pig gastrointestinal tract had a recovery of 8.02%. In another report same chromatographic techniques were used to purify bacteriocin from strain *Bacillus subtilis* isolated from gut of *Labeorohita* which had specific activity of 1453.27 AU/mg and yield of 9.8% [37].

After final purification steps, a single band of 31.2kDa in SDS-PAGE was found. Similarly, Valdes-Stauber and Scherer [38] produced a bacteriocin, linocin M18 from *Brevibacterium linens* which has molecular mass of 31 kDa. An and coauthors [39] produced a bacteriocinCAMT2 from *Bacillus*

amyloliquefaciens isolated from marine fish *Epinephelus areolatus* which has molecular mass of 20 kDa. Sharma et al., reported a bacteriocin produced from *Bacillus subtilis* R75, which has molecular weight of 12 kDa[22]. Another study conducted by Banerjee et al., isolated bacteriocin producing *Bacillus subtilis* LR1 from gastrointestinal tract of fish showed 50 kDa protein and [37]. Some of the bacteriocins produced by *Bacillus* are identical to lactic acid bacteria but still the *Bacillus* bacteriocin classification system is lagging behind as compared to lactic acid bacteria [40].

Biochemical characterization of purified bacteriocin

Purified bacteriocin was then subjected to multiple biochemical characterizations. Effect of different temperatures ranging from 30°C to 121°C on the residual activity of bacteriocin was determined. Stability studies inferred that the bacteriocin was stable over a wide range of temperatures of 30°C to 70°C but it drastically reduced at 100°C and 121°C.

The behavior of bacteriocins towards heat-resistant pathogens also varies [22]. Bacteriocin produced from *Brevibacterium linens* was active at 40-50°C for 30 minutes [38]. However, RX7 bacteriocin isolated from *Bacillus amyloliquefaciens* was active upto 100°C for 30 minutes [41]. While studying the effect of pH on residual activity, the purified bacteriocin from *Bacillus subtilis* VS was found to be stable over broad pH range of 4 to 8 but it was highly affected at extreme pH values of 3 and 9 (Figure 5). Bacteriocin from *Brevibacterium linens* was stable upto pH 9 [38]. Whereas Salazar et al., produced two bacteriocins from *Bacillus amyloliquefaciens* were found stable at pH 2-10 [42].But BLISm6c and BLISm387 bacteriocin like compounds produced from *Bacillus cereus* was stable at pH ranges from 3-7[43]. So, the study suggested that bacteriocin from *Bacillus subtilis*VS is stable at wide temperature as well as pH ranges of the several proteases assayed to check their action on bacteriocin; all of them showed positive results. The results suggested that as it was hydrolyzed by all proteolytic enzymes tested which confirms the proteinaceous nature of bacteriocin. All these attributes of the bacteriocin from *Bacillus subtilis* under study suggests that it can be potentially exploited for preservation of various foods. Similar results were observed with other bacteriocins also [22, 41] which confirms that it is peptide in nature; therefore, it can be degraded by gastric juices, making it safe for human consumption.

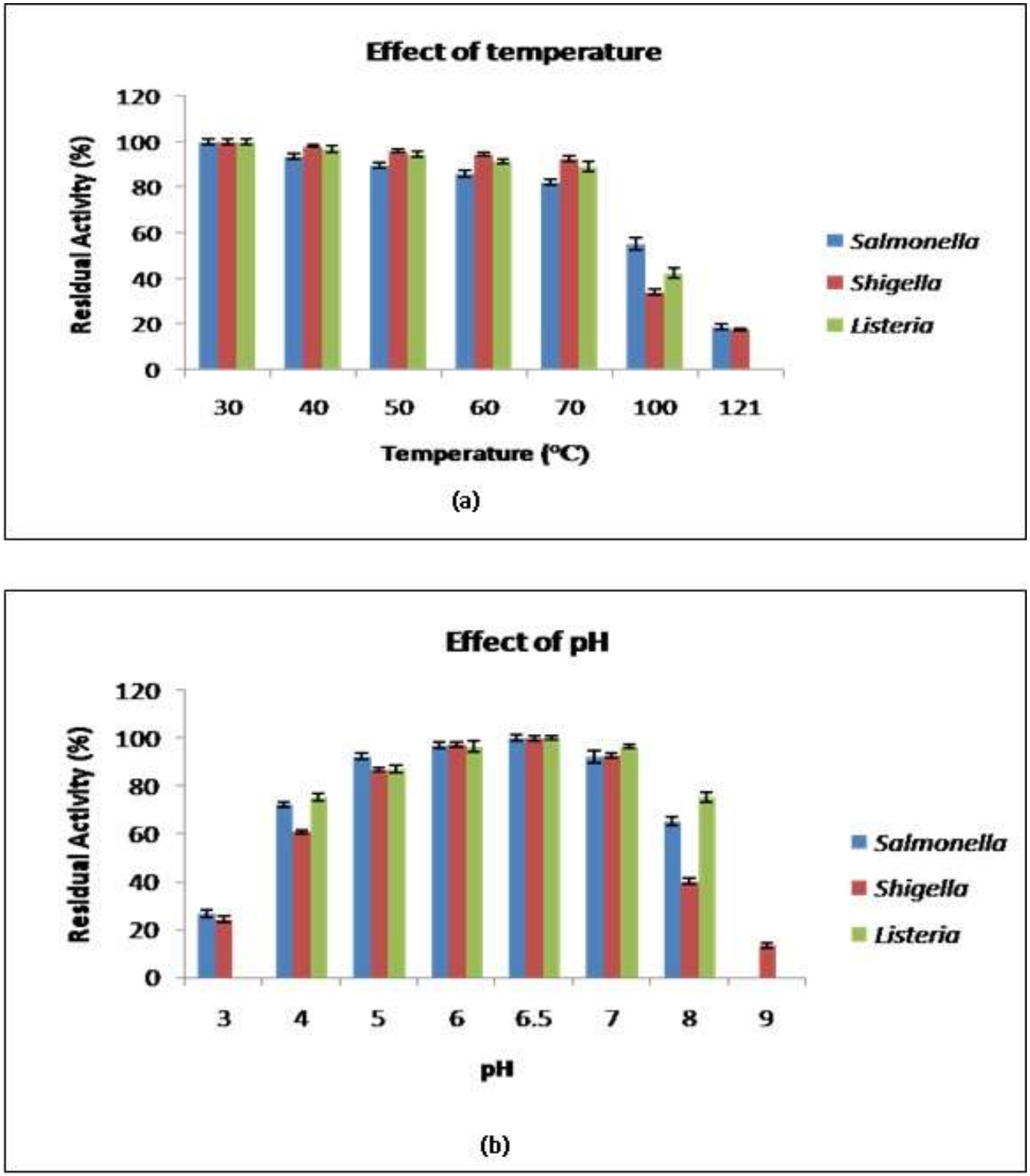

Figure 5. Effect of temperature and pH on the stability of bacteriocin from Bacillus subtilis VS. Mean error bars represent the mean (n = 3) ± standard deviation.

CONCLUSION

In this study, *Bacillus subtilis* VS, a strain derived from the mango was found to produce a potent bacteriocin. It was found to be potentially active against various pathogens like *Shigellaboydii*, *Salmonella typhi*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus.* Employing multiple chromatographic techniques bacteriocinwas purified to homogenecity and its molecular weight was found to be 31.2 kDa. Purified bacteriocinwas found to be stable over broad range of pH as well as temperatures. Its proteinaceous nature was confirmed by hydrolysing it with various proteases. All these potential attributes of bacteriocin from *Bacillus subtilis* VS opens an avenue for its use in various food industries.

Conflicts of Interest: Declare conflicts of interest or state “The authors declare no conflict of interest.”

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