

Research Article

Antagonistic effect of bacteriocin from *Bacillus subtilis* against food-borne pathogens

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Abstract

Preservation of food items through natural and biological methods has become a norm in order to prevent huge financial losses that may be incurred as a result of contamination of raw foods and/or food products. Biological preservation methods help avert the chances of food born ailments thus ensuring the fulfillment of ever-increasing global food requirements. Growing skepticism among the customers in recent years regarding food safety and preservatives used in food industry has fetched remarkable consideration to the use of *Bacillus* bacteriocin in food processing as well as human health care. *Bacillus subtilis* was isolated from soil samples for determination of its microscopic and biochemical characteristics. The proteinaceous antimicrobial compounds were extracted through ammonium sulfate precipitation method. The bacteriocin was partially purified through SDS-PAGE; optimization of bacteriocin at different pH levels, heating temperatures and storage conditions was performed. Indicator strains were procured from CMS, UAF, Faisalabad. The antimicrobial assays of extracted bacteriocin were determined on Mueller-Hinton media through agar well diffusion method. The extracted bacteriocin showed comparatively better antibacterial activity against indicator *Staphylococcus aureus* followed by *E. coli* and *Salmonella*. Maximum activity was observed at pH 7, heating temperature 37°C and storage 4°C, comparatively higher activity against Gram positive bacteria was noticed.

Keywords: Biological preservation; Economic losses; Antimicrobial compounds; Optimization

Introduction

The economy of food processing units has been hit hard by the emergence of noxious pathogens in food stuffs. Loss of customer trust in food industries has resulted in health scares as a direct consequence to consumption of spoiled or contaminated

food. Despite advances in food preservation technologies, and heterogeneity in food processing and raw materials, the smartness of microbes has enabled them to evade food safety measures with growing ease, causing food spoilage, which is one of the leading concerns for the food industry in present

times [1, 2]. Estimations reveal that more than 200 lethal diseases are transmitted through food spoiled by microbes [3]. *Campylobacter*, *Yersinia*, *Staphylococcus*, *Escherichia coli*, *Salmonella* and *Listeria* are considered the major bacterial food-borne microbes these days [4]. Serious threats modeled by antibiotic-resistant food borne bacteria and fungi have increased the urgency for use of substitute therapeutics. Antimicrobial peptides (AMPs) are regarded as highly promising therapeutic antimicrobial compounds [5]. Principally microbes compete for limited space and nutrients in their particular type of environments, which is why they produce special type of compounds including proteins, bioactive peptides, antibiotics and bacteriocin [6]. Bacteriocin is ribosomally synthesized bioactive peptide comprising about 30-60 amino acids produced by both Gram negative and Gram positive bacteria. These are usually, but not always, effective against most closely related strains of the producer strains [7, 8].

Bacillus species are outstanding producers of antimicrobial compounds such as peptides, antibiotics and bacteriocin. Bacteriocin production from *Bacillus* is considered as second most important production after *Lactic Acid bacteria* (LAB). They not only produce ample masses of bacteriocin, which is ribosomally synthesized, but also some non-ribosomally produced peptides which are bacteriocin-like inhibitory-substances (BLIS), genetically non-characterized peptides. So *Bacilli* are prolific in producing true bacteriocins and BLIS as well [9, 10]. The bacteriocins from *Bacilli* have a great potential preserving application in many food compounds like in dairy foods such as milk and cheese [11, 12]. Bacteriocin performs either bactericidal or bacteriostatic activity. The diversity in the performance and the decision as to which action will be

performed is affected by too many physical factors such as quantity of extracted bacteriocins, the methods of purification of extracted bacteriocins and the physiochemical properties of target cells, which are mainly considered sensitive [13].

The Bacteriocin of *Bacillus subtilis* has been anticipated as an auspicious alternative of the prevailing antibiotics for the treatment of food-borne diseases for it is low-priced as well as non-hazardous nature vis-à-vis human and animal health [14].

Certain readings have assessed the therapeutic effectiveness of bacteriocins in the handling of animal and human diseases caused through food spoilage because of the resistance shown by resilient microbes to conventional antibiotics. One of the advantages of using bacteriocin in food industries is their productions at reasonably lower cost, which makes the bacteriocin an excellent candidate for antimicrobial compound and also an effective growth promoter in farm and industrial environments. The proteinaceous nature infers a putative degrading action in the gastric and intestinal tracts of animals and human beings, signifying their use as accepted preservers in foods [7]. Keenly observing the dreadful harms of food handling units and their frightening influences on human health, the present study has been defined. To extract bacteriocin from *Bacillus subtilis* isolated from soil sources and evaluate the antagonistic effect of bacteriocin in different food degrading bacteria.

Materials and methods

Collection and isolation of *Bacillus subtilis* from soil

A total of 30 samples (approximately 10 g from each site) were collected after removing upper 10 cm of litter leaf from different areas of Faisalabad using clean, dry and sterile polythene bags along with sterile spatula. All the samples were immediately

transferred to laboratory. For further processing of samples and isolation of bacteria these were stored at 4°C [15].

Inoculation and incubation

About 5 gram of soil from each sample was mixed with sterile water homogenized and heated at 80°C and allowed to stand for 1 h at room temperature. A volume 0.5 ml of this suspension was inoculated into nutrient broth and incubated for 48 hours. Microbial growth observed by turbidity and aliquots was inoculated onto nutrient agar plate and was incubated at 37°C for 24 hours [16].

Purification of bacterial colonies

Purification was done using streak plate method by transferring colonies from Nutrient agar plates to other Nutrient agar plates until pure colonies were obtained.

Identification of isolates

The purified streaks of bacteria were presumptively identified by cultural characteristics such as color, texture shape and size of colonies. Morphological characteristics were noticed after Gram staining, spore staining and acid fast staining. The isolates were run through couple of biochemical tests. Catalase test, Methyl-Red test, Indole production, Voges-Proskauer, Starch hydrolysis and sugar fermentation tests were executed and the isolated organisms from the sample were screened on the basis of their morphological and biochemical characteristics as described in Bergey's Manual of systematic bacteriology [17].

Extraction and partial purification of bacteriocin

Due to their proteinaceous nature, bacteriocin was concentrated through the application of salting-out methods, being ammonium sulfate the most frequently used salt [18]. The solid salt was added to the distilled water slowly until the desired saturation percentage of ammonium sulfate was reached. A 36-h-old culture of the bacteriocinogenic *B. subtilis* strain was

centrifuged at 8000 rpm for 25 min at 4°C. The peptidic fraction was precipitated from the cell-free supernatant with 40% saturated ammonium sulfate. The suspension was agitated with magnetic stirrer and incubated overnight at 4°C. Salted-out proteins were further precipitated by centrifugation at 15000 rpm for 20 minutes. Collected pellets were dissolved in a small volume of 10 mM phosphate buffer (pH 7.0) or distilled water. The bacteriocin was partially purified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Stability of isolated bacteriocins

Stability as well as antimicrobial potential of isolated bacteriocins were checked at different (3, 5, 7 and 9) pH levels, heating temperatures (27°C, 37°C, 60 °C, and 80°C for 40 min) and different storage temperatures (-20°C, 4 °C and 37°C).

Pathogenic bacterial strains

The cultures of pathogenic strains (*Staphylococcus aureus*, *E. coli* and *Salmonella enteritidis*) were obtained from stock culture of the Department of Clinical, Medicine and surgery, Agriculture University, Faisalabad. Cultures used in this study were transferred twice in to the blood agar medium before use.

Determination of antibacterial assay

The antibacterial activity of bacteriocin was detected against *Escherichia coli*, *Salmonella enteritidis* and *Staphylococcus aureus* by agar well diffusion method. Bacteriocin (25µl) treated at different storage temperatures, thermal conditions and pH values were added to 6 mm wells on Mueller-Hinton agar plates. Mueller-Hinton plates were spread with 100 µl suspension of each indicator strain containing 2 x 10⁸ cfu/ml [19]. The plates were incubated for 24 hours at specific temperature according to indicator strains used. The diameters of zone of inhibition were measured using vernier caliper.

Statistical analysis

Data was analyzed by applying Standard Error (Mean \pm SE) and analysis of variance (ANOVA) under completely randomized design.

Results

The genus *Bacillus* is basically a heterogeneous group of microbes belonging to Gram positive species of bacteria. These are endospore-forming Gram positive rod-shaped aerobes including extremophiles and mesophiles. These are characterized by catalase production. The *Bacilli* have colonized diversified environments due to their ability of withstanding even harsh environments assisted by formation of resistant endospores [20].

In the present investigation, *Bacillus subtilis* was isolated from soil appeared as yellowish white colonies with spreading type of growth. Round and irregular colonies with diameter of 2-4 mm, Circular form and Flat elevation were observed on Nutrient agar plates. Gram staining showed purple color rods arranged separately and in chains. Culture was stored in nutrient broth at -20°C with 10% (v/v) glycerol addition. The bacterium was identified on the basis of morphological and biochemical characteristics given in Table 1. Bacteriocin extracted from nutrient broth culture of *Bacillus subtilis* was stored in pellet form at 4°C in PBS [26]. SDS-PAGE gave the molecular weight of extracted bacteriocin which was in the range of 3-6KDa [9].

The antimicrobial activity of extracted bacteriocin against food degrading microbes was determined by agar well diffusion method on Mueller-Hinton medium through measuring the diameters of zones inhibited by microbes (Table 2, 3 and 4) in mm by vernier calipers [21]. The antagonistic activity in arbitrary units per ml (Au/ml) as a measure of bacteriocin production was calculated (Table 5, 6 and 7) as described by Bhuvanewari and his co-workers [22].

Results indicated that bacteriocin extracted from *Bacillus subtilis* showed maximum zone of inhibition against *Staphylococcus aureus* followed by *E. coli* and *Salmonella enteritidis* [22]. Bacteriocin showed maximum activity at pH 7. Partial reduction in activity was observed with shift of pH from neutral to alkalinity and acidity. The production of bacteriocin increased with increase in temperature but at 80°C, there was an abrupt decline in activity. The activity of bacteriocin was maximum when stored at 4°C. A slightly reduced yet significant activity was observed at -20°C suggesting its role as a preservative even at chilling temperatures.

Statistical analysis showed that there was a significant difference in means of zone of inhibition produced by bacteriocin against different food- spoiling microbes at different levels of pH, heating temperatures and storage conditions.

Table 1. Morphological and biochemical characteristics of isolated bacteria

S. No	Gram staining	Catalase test	Motility	Starch hydrolysis test	Indole test	MR	VP	Glucose fermentation	Maltose fermentation
1.	+ve rods	+ve	Motile	+ve, zone of clearance	-ve	-ve	+ve	+ve (Gas produced)	+ve (Gas produced)

Table 2. Zone of inhibition (mm) and OD values of bacteriocin at different pH levels produced against food pathogens

Treatment	<i>Staphylococcus Aureus</i>	<i>E. coli</i>	<i>Salmonella enteritidis</i>	OD at 600 nm
pH				
3	9.4	8.9	8.4	0.95
5	9.8	9.4	9.1	1.31
7	13.9	13.0	12.4	1.52
9	8.8	8.4	8.2	0.83

Table 3. Zone of inhibition (mm) and OD values of bacteriocin at different heating temperatures produced against food pathogens

Treatment	<i>Staphylococcus aureus</i>	<i>E. coli</i>	<i>Salmonella enteritidis</i>	OD at 600 nm
Heat(° C)				
27	10.2	9.8	8.5	0.95
37	11.6	10.1	9.4	1.31
60	7.1	6.8	6.2	1.52
80	1.4	1.2	1.1	0.83

Table 4. Zone of inhibition (mm) and OD values of bacteriocin at different storage temperatures produced against food pathogens

Treatment	<i>Staphylococcus aureus</i>	<i>E. coli</i>	<i>Salmonella enteritidis</i>	OD at 600 nm
Storage temperature (° C)				
37	11.2	9.6	8.1	0.95
4	11.6	10.1	9.4	1.31
-20	10.1	9.1	7.1	1.52

Discussions

Massive illnesses caused by food pathogens and a growing consumer refusal for the foods have urged scientists to look for novel antimicrobials to reduce food-borne health scares. The purpose of this study was to isolate bacteriocin producing *Bacillus subtilis* locally from soil source and to screen their bacteriocin as natural bio preservative and antimicrobial agent against Gram positive, Gram negative bacteria which produce food spoilage. *Bacillus subtilis* isolated from different soil samples were characterized and subjected to study the antimicrobial and food preserving activity of their bacteriocin.

Studies suggest that *Bacillus* species are outstanding producers of antimicrobial compounds such as peptides, antibiotics and bacteriocin. Bacteriocin production from *Bacillus* is considered as second most

important production after *Lactic acid bacteria* [23]. Basically bacteriocins are polypeptides synthesized ribosomally produced by different microorganisms which belong to the eubacteria taxonomic tree [24]. Bacteriocin was isolated in little amounts due to their adsorption to cell surface. These were isolated by precipitation with ammonium sulfate according to Sambrook and co-workers [18]. This method recovered only a small portion of bacteriocin present in broth due to absorption to cell surface [25]. The Pellets collected were stored at 4°C in PBS [26]. The bacteriocin was partially purified and molecular weight was determined through SDS-PAGE. Previously, Nivedita with co-scientists extracted and purified *Bacillus subtilis* bacteriocin by gel exclusion and estimated molecular weights by SDS-PAGE. They

found that activity units of bacteriocin increase with each step of purification [27]. Bacteriocin extracted from *Bacillus subtilis* exhibits remarkable action at wide ranges of pH, nutrients, heating temperatures, enzymes and storage conditions. Bacteriocin optimized at different independent parameters showed variation in activity units produced in zones inhibited which suggested that with increase in bacteriocin production (Au/ml), the diameter of zone of inhibition gets widened [28]. Production of bacteriocin and maintenance is normally affected by diverse environmental influences such as distinctive atmospheric growth conditions, ultrasound shock and nutrient depletion on bacteriocin adsorption from cell [29].

The spectrum of inhibition was different for each bacteria used as indicator strains. The results indicated that *Staphylococcus aureus* was most sensitive to bacteriocin extracted from *Bacillus subtilis* followed by *E. coli* and *Salmonella enteritidis* (Figure 1, 2, 3). Recently potential antagonistic effects of Bacteriocins of *Bacillus subtilis* BMPO1 were determined which showed maximum activity against *Staphylococcus aureus* followed by *E. coli* [22].

Studies conducted before clearly indicated that bacteriocin isolated from *Bacillus* bacteria shows remarkable inhibitory activities against Gram positive, Gram negative and food spoiling Fungi [30]. The maximum inhibition of *Staphylococcus aureus* signifies the fact the bacteriocin is effective against the most closely related strain of the producer strain.

The variations of sensitivity observed against different microbes are due to specific genetic coding of target strains and characteristics of bacteriocins treated at different independent variables. Nisin was the first finest studied illustrative of the Lantibiotics finding special applications in the inhibition effect of late-blowing of cheese by hindering the development of

spores *Clostridium* and was used in selected pasteurized food products [8].

Sutyak reported that subtilocin produced by *Bacillus subtilis* 22 isolated from fermented Chinese soybeans showed greater potential of antibacterial activity against food-borne resistant pathogens like *Staphylococcus aureus*, *Salmonella typhimurium*, *Listeria monocytogenes* and other deadly bacterial and fungal disease causing microbes [31].

The results of critical trial of statistical analysis indicated that there was significant difference between means of zones inhibited by bacteriocin optimized at different levels of pH and heating temperature. With shift of pH from acidic to neutral, the production and activity of bacteriocin increased but from neutral towards alkalinity, partial reduction of activity was noticed. Yet the unique property of bacteriocin of *Bacillus subtilis* of tolerating wide pH range places it as potential bio preservatives of acidic, neutral and alkaline foods [32].

Bacteriocin showed maximum activity when stored at 4°C and a slight reduction of activity was seen on 37°C and -20°C (Figure 3). Scientists through experimental results suggested its use as natural preservatives of foods processed even in chilled conditions [26].

The activity of bacteriocin was found good even at 27°C heating suggesting its role as preservatives of foods stored at room temperatures. With certain increase in temperature, the production of bacteriocin declined and an abrupt change of activity was seen for further increase in temperature from 60°C to 80°C (Figure 2). Previously bacteriocin from 8A strain of *Bacillus* species was purified and scientists concluded that this bioactive antimicrobial compound was stable at 55°C but lost its activity at 87° C [16].

Bacteriocin extracted from *B. subtilis* holds optimistic repute being used as bio preservative of food owing to all the

necessary characteristics which prevent spoilage process. Thus preservation of food stuffs by either natural or microbiological strategies can be regarded as acceptable approach to lessen the occurrence of food

borne ailments, resolving fiscal losses due to spoilage of raw food items with microbes, and develop better food supplies exactly according to the needs of rising world population [33].

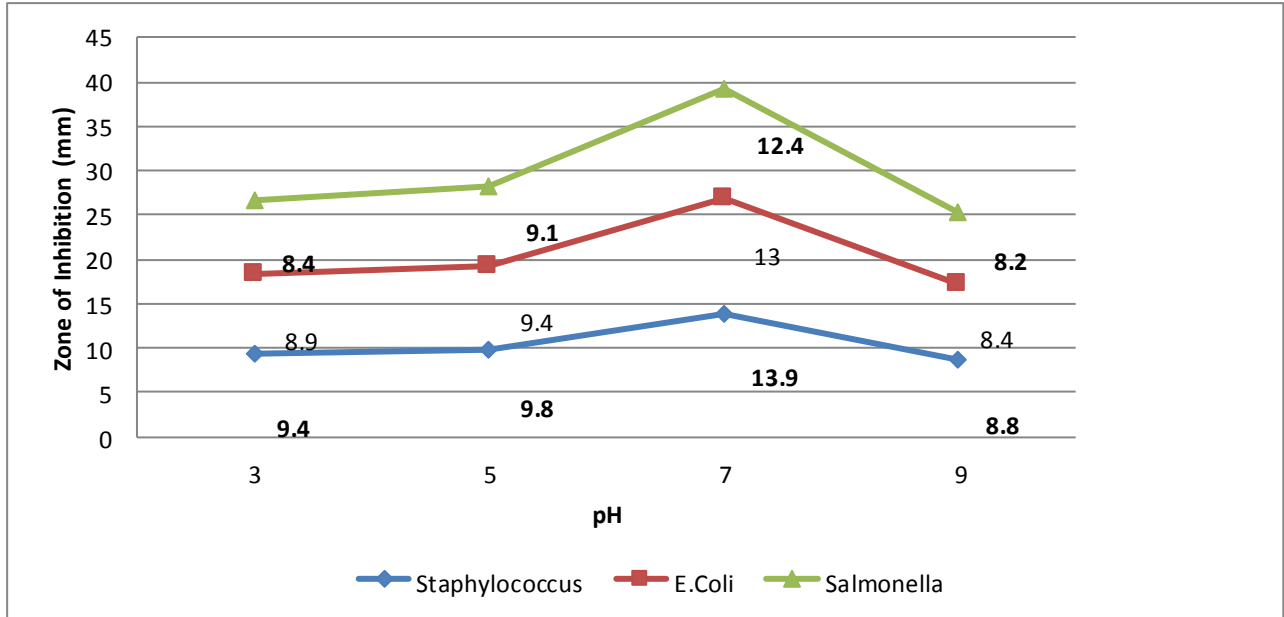


Figure 1. Comparison of antibacterial effects of bacteriocin against food pathogens at different pH levels

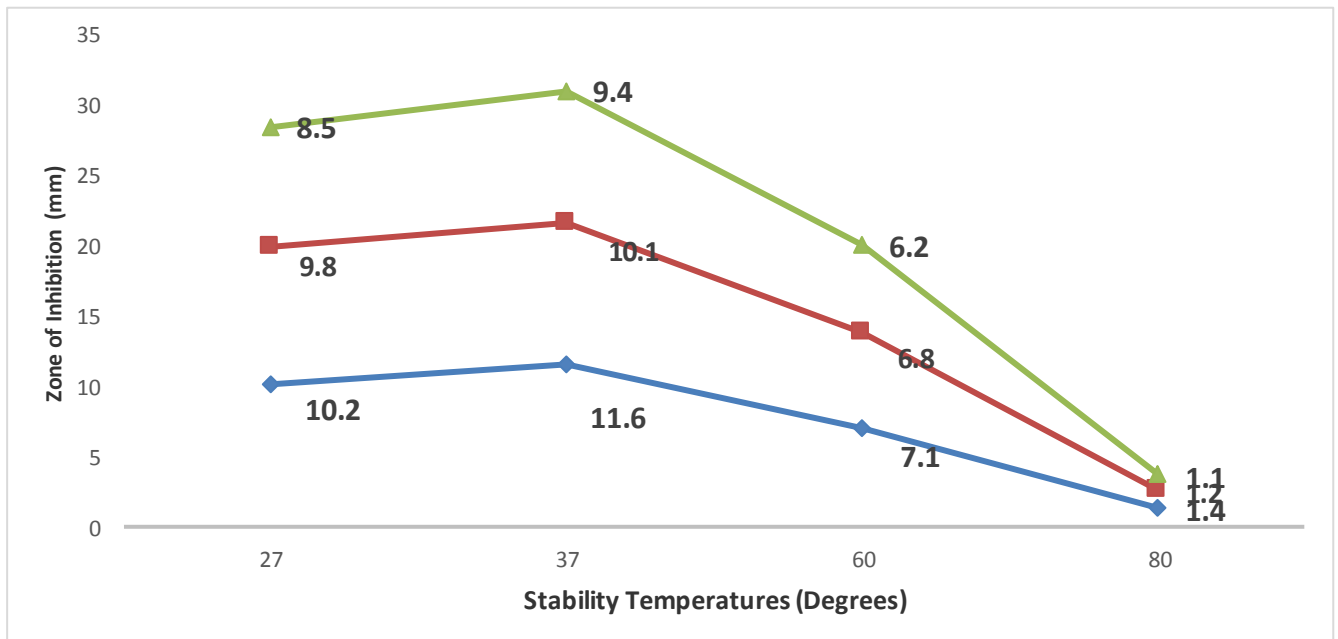


Figure 2. Comparisons of antibacterial effects of bacteriocin against pathogens at different heating temperature

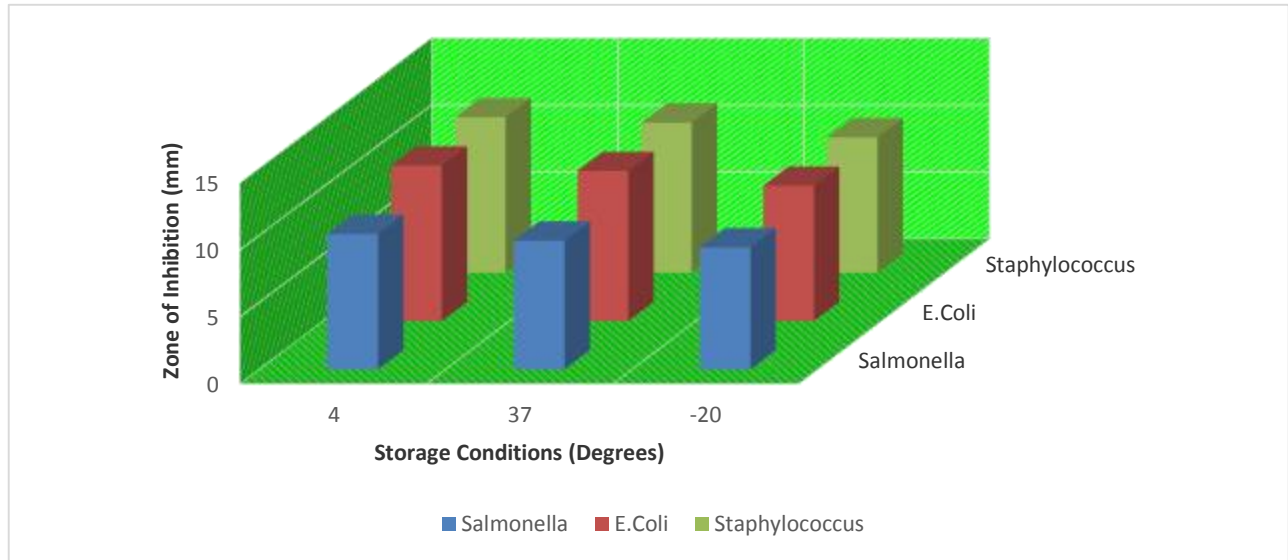


Figure 3. Comparisons of antibacterial effects of bacteriocin against pathogens at different storage temperatures

Conclusion

Bacteriocin extracted from *Bacillus subtilis* exhibited remarkable antibacterial activity. Maximum activity was exhibited against Gram positive bacteria (*Staphylococcus aureus*) followed by Gram negative bacteria (*E. coli* and *Salmonella enteritidis*). The Highest activity of bacteriocin was observed at pH 7, heating 37°C and storage 4°C. These results aptly show that bacteriocin extracted from *Bacillus subtilis* is good at inhibiting growth of many pathogenic microbes so they can be safely used as natural preservatives for foods and as substitute of conventional antibiotics in therapeutics.

Authors' contributions

Conceived and designed the experiments: SN Hussain & M Ashraf, Performed the experiment: SN Hussain, Analyzed the data: SN Hussain, Contributed reagents/materials/analysis tools: M Ashraf, M Jamil & H Hanif, Wrote the paper: SN Hussain.

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