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PRODUCTION AND THERAPEUTIC POTENTIAL OF BACTERIOCIN PRODUCED BY INDIGENOUS ISOLATES OF *Bacillus subtilis*

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Bacteriocins are the antimicrobial peptides that are produced by many of the bacterial species as secondary metabolites. They have potential to inhibit the growth of many other pathogenic bacteria. The present study was conducted to check production and therapeutic potential of bacteriocin produced by different indigenous isolates of *Bacillus subtilis*. Soil samples were collected from different environments including soil mud, agricultural land soil, sewage, and deteriorating plant matter. These samples were cultured on Nutrient Agar for isolation of *B. subtilis*. These isolates were further purified by culturing and identified by biochemical.16SrDNA technique was used to confirm the *B. subtilis*. The confirmed isolates were evaluated for their potential to produce bacteriocin using agar well diffusion method. Bacteriocin was purified using Ammonium sulfate precipitation method, and its chemotherapeutic properties were checked by developing two models, Superficial skin model and experimental Gram negative septicemia model. The results indicated that *B. subtilis* isolates showed a band of 1311bp on gel in 16SrDNA analysis and sequencing also confirmed *B. subtilis* isolates. No toxic effect of bacteriocin was observed in experimental animals. There was no mortality in treated and controlled group animals during experimentation. In conclusion, bacteriocin peptides have ability to inhibit bacterial growth and may be exploited to treat microbial infections.
Keywords: Bacteriocin, Secondary metabolites, Toxic effect, 16SrDNA, Indigenous.

INTRODUCTION

The rapid increase and spread of drug resistance pathogens have constrained the thought in scientists to exchange strategies for battling contaminations and infections. One big limitation of utilizing wide spectrum antibacterial agents is that these almost kill any type of bacteria which do not particularly resist a drug. The drugs which kill a wide range of bacteria create drug resistance in both pathogenic bacteria and commensals microorganisms (Walker and Levy, 2001). Antimicrobial substances produced by microorganisms have changed the microbial ecology. Bacteriocins are secondary metabolites and belong to class of antimicrobial substances produced by a variety of bacteria. They are robisomally-synthesized proteins or peptides and have antibacterial action (Gálvez *et al.*,2007). Bacteriocins show antimicrobial action often against closely associated bacterial species (Cladera-Olivera *et al.,* 2004). Bacteriocins have great variation even within same species of bacteria (Motta and Brandelli, 2008). Bacteriocins are also defined as biologically active peptides with bactericidal mode of action. They include a variety of protein molecules having different size, activity, immune mechanisms as well as different antimicrobial targets (Heng *et al.,* 2007). As compared to traditional antibiotic compounds, they are different from them in one special way that they are narrow spectrum and kill only those bacteria which are closely related to each other (Riley and Wertz,2002).

Most of the species from family Bacillus are likewise considered as GRAS bacteria and they are critical bacteriocin producers (Martirani *et al.,* 2002). Bacillus group bacteria are known for the production of various antimicrobial substances like peptides, lipopeptides, antibiotics, and as well as bacteriocins. There many species of *Bacillus* genus that have ability to produce a variety of useful antimicrobial products like bacteriocins and among them *B. subtilis* is the most important one. This species is considered nonpathogenic and non-toxic to humans as well as plants to cause disease (Stein, 2005). The bacteriocins produced by one species of bacteria can be applied as antibacterial against other related bacteria. For example, bacteriocins produced by *B. subtilis* have been proved experimentally to be used against *Listeria monocytogenes* infection (Sabaté and Audisio, 2013).Almost 90% bacteria have ability to produce bacteriocin products and many of these have broad spectrum inhibitory activity for different microbes (Bizani and Brandelli, 2002).The current research was performed to check potential of *B. subtilis* bacterium for bacteriocin production isolated from soil samples and its toxicity evaluation in experimental animals.

MATERIALS AND METHODS

Isolation of bacteria: *Bacillus subtilis* was isolated from different environments including soil mud, agricultural land soil, sewage, and deteriorating plant matter. Samples(n=50) were inoculated in nutrient agar and incubated at 37 °C for 24 hrs. Each soil sample (1 gram) was mixed with phosphate buffer saline (PBS)(MP Biomedicals®, St. Ana, USA) to make the total volume up to 10 ml in test tubes separately, vortexed to homogenize, and heated at 80°C in a water bath for 10 minutes then cooled at room temperature. A total of 500 µl of soil clay was added to 10 ml of nutrient broth (Oxoid®, Hampshire, UK) and incubated at 37 °C for 24 hours. Then a total of 200 µl of each test tube of nutrient broth was spread on Petri plates having nutrient agar (Oxoid®, Hampshire, UK). These Petri plates were given incubation for 24 hours at 37°C. After incubation, the growth of bacteria was noticed with respect to shape, size color, and texture. Again, the colonies were streaked on nutrient agar plates for getting the pure cultures (Bizani and Brandelli, 2002).

***Identification of isolates*:** Isolated bacterial colonies were identified based on culture characteristics such as color, shape, surface size and texture of colonies. Microscopic characteristics were studied after Gram staining and spore staining. Different biochemical tests including; methyl-Red, catalase test, H_2S production, indole production, hydrolysis of starch, Voges-Proskauer and citrate utilization test were performed following Bergey's Manual of Determinative Bacteriology (Brown, 1939).

***Molecular characterization*:** For confirmation, 16S ribosomal DNA based PCR was performed. The DNA was extracted from isolated *B. subtilis* and 16S ribosomal DNA was amplified (Table 1) and sequenced (Rahman *et al.,* 2014).

***Screening of isolates for bacteriocinogenic potential*:** Agar well diffusion assay was utilized for the determination of bacteriocinogenic potential of isolates (Jack*et al.,* 1995).A uniform lawn of indicator bacteria(*Staphylococcus aureus* ATCC 29923 and *E. coli* ATCC 29922) was prepared in normal saline (0.5 MacFarland indexed) and spread on nutrient agar with sterile cotton and wells of uniform width (8 mm) were made. The cell culture supernatants ((50 µl)) from isolated bacteria was dispensed in each well while the central well was poured with broth only and kept as negative control. Then plates were incubated at 37°C for 24 hours. The zones of inhibition around each well were observed and measured. B. *subtilis* strain with antibacterial activity was chosen as a 'bacteriocin producer' and was utilized for further studies (Hu, Y. *et al.,* 2017).

Elimination of non-bacteriocinogenic isolates:

a) Inhibitory action due to lytic bacteriophages: Reverse side agar technique was utilized to examine if the inhibitory activity is because of bacteriocin or bacteriophages (Parrot *et al.,* 1989). The presence of a clear zone of inhibition around the stabbed culture would exclude the possibility of inhibitory activity due to lytic phages and would be taken as a sign that inhibition is because of bacteriocin.

b) Inhibition of the indicator organism due to protease production: The Cell Free Supernatant (CFS) will be blended with protease inhibitors like ethylene diamine tetra-acetic acid (EDTA) with the ratio of 1:1 and the inhibitory activity will be observed by agar well diffusion technique (Eftekhar *et al.,* 2003).

Extraction and purification of bacteriocin from isolated Bacillus: Cell-free culture (CFS) supernatant was obtained by growing producer strain in nutrient broth in a shaking incubator at the rate of 120 rpm at 37°C until log phase of bacteria. Cells were reaped by centrifuging at 8000×g for 10 mins and pellet was disposed of. The membrane sterilization of CFS was done by passing it through 0.45-µm and 0.25-µm sized pores, purified and stored at -20^{o}C until use (Zheng and Slavik, 1999).

The extracted bacteriocin was partially purified by the Ammonium sulfate precipitation method. Appropriate concentration (w/v) of ammonium sulfate was added to accomplish 40%, 60% and 80% concentration of ammonium sulfate $(NH_4)_2SO_4$ saturation at 4^{o}C. The precipitated protein was isolated by centrifugation at 8000×g for 30 minutes at 4^{o}C. The pellet obtained from the ammonium sulfate precipitation was dialyzed in a molecular weight cut-off tubular cellulose membrane against 2000 ml sodium citrate buffer for 24 hours with two changes. After dialysis, the purified sample was collected in sterile tubes.

Quantification of bacteriocin: The amount of partially purified protein obtained from the dialysate was estimated by the method as described by Bradford.

The standard conditions were made according to 1ml standard assay using a protein amount of 0, 125, 250, 500, 750, 1000 and 1500 µg/ml. A volume of 20 µl of sample and standard (in separate Eppendorf tubes) were taken and ambient temperature equilibrated 1X dye reagent (1ml) was added and absorbance at 595 nm of standards and samples was measured against the blank sample. The Bacteriocin quantification was determined by comparing OD595 of sample with that of standard curve plotted for OD595 (Bradford, 1976).

Table 1. Sequence of primers used for *B. subtilis* in PCR identification.

Primer	Sequence	Product size	Reference
EN1F	5′-CCAGTAGCCAAGAATGGCCAGC-3′	(103–124 bp)	Ashe *et al.,* (2014)
EN1R	5′-GGAATAATCGCCGCTTTGTGC-3′)	(1,413–1,393 bp)	Ashe *et al.,* (2014)

Calculation of LD_{50} of bacteriocin: A total of 20 mice having an average weight of 20-25 g were kept in the animal house of Institute of Microbiology, UAF and were divided into four groups (5 mice / group). The group1st was kept as control group (injected 0.4 ml sterile saline). While the other three groups; 2nd, 3rd & 4th were injected 0.25 g/ml, 0.5 g/ml and 1.0 g/ml of bacteriocin respectively through intraperitoneal route. Read and Munch method was followed for calculation of LD_{50}(Marlida *et al*., 2016).

Animals were examined day by day for symptoms, skin allergy/dermonecrotic response & mortality rate for 14 days. The blood was collected for biochemical profiling and histopathological changes were observed in different organs such as heart, liver, and kidney (Rijun, 2001).

***Chemotherapeutic Aspects of Bacteriocin*:** The chemotherapeutic aspects of bacteriocin were determined by developing two models:

a) Superficial skin model (Kugelberg *et al.,* 2005)
b) Experimental Gram- negative septicemia (Corrigan and Kiernat, 1975)

***a) Superficial skin model*:** A total of twenty specific pathogens free (SPF) rabbits were procured having an average weight of 3 kg and divided into four groups (each with n=5). Group A animals were inoculated with *Staphylococcus. aureus* ($5x10^7$CFU/mice) through skin after damaging epidermal layer of skin (topical route) on the both flanks of rabbit. After 4 hrs of this injection, bacteriocin (1.0 g/l) was given orally. Group B was kept as control group without inoculation of bacteriocin without infection. Animals in group C were challenged with *S. aureus* but this group was kept as controlled infection group without inoculation of bacteriocin. Group D animals were treated with commercially available Gentamycin Sulphate ointment on injured skin. The treatment procedure was repeated twice for four days. The skin injuries of the experimental animals were checked for the presence of clinical signs for 24 hours after experimental treatments.

***b) Experimental Gram- negative septicemia model*:** The experimental rabbits (n=20) were divided into five groups each with four rabbits and inoculated with *Escherichia coli* (*E. coli*) (2×10^{10} CFU/kg of body weight) through Intraperitoneal route. Rabbits in group 1 were administrated with isolated bacteriocin (1.0 g/l) 90 minutes before inoculation of *E. coli*). Group 2 and group 3 rabbits were given only purified bacteriocin injection after 6 hours & 3 hour of post injection of *E. coli* respectively. Group 4 rabbits were kept as positive control injected with *E. coli* only while group 5 was inoculated with commercially available Gentamycin antibiotic after 6 hrs of *E. coli* injection. The animals were observed regularly for appearance of clinical signs and symptoms including sickness.

Toxicological Studies: Blood was utilized for hematological investigations. Results relating to thrombocytopenia & leucopenia were observed. Antibody production was also checked against the given bacteriocin using serum collected from all the experimental groups. Other parameters relating to toxic indices including respiratory distress, depression diarrhea, and vomiting were recorded.

Statistical Analysis: The data obtained was analyzed statistically using analysis of variance.

RESULTS

***Isolation and purification of B. subtilis isolates*:** The presumptive samples for *Bacillus* further processed on nutrient agar plates by streak plate method showed colonies which were large in size (3 mm) flat, rhizoidal, undulated and fuzzy white and some were slightly yellow colored like that of *B. subtilis* bacteria.

It was found that out of total 50 samples, 22 samples were found to be presumptively positive for *B. subtilis.* Among twenty-two isolates, sample BS1, BS2, BS16, BS18, BS19, BS20, BS31, BS35, BS36, and BS37 showed fuzzy white color growth on media while the rest of the isolates gave slightly yellow color colonies (Fig.1).

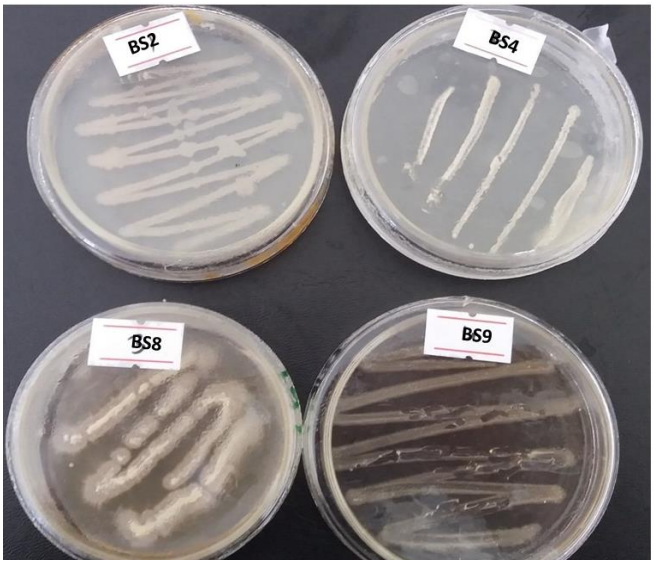

Figure 1. *B. subtilis* isolates on nutrient agar; fuzzy white color growth was observed

Microscopic and biochemical confirmation: All the positive isolates were appeared as Gram positive rod shaped with single cell or clump cell arrangement (Fig. 2). All the 22 isolates were positive for Voges-Proskaeur, oxidase, Simon citrate utilization and catalase test. All isolates were negative for Indole production test. All the isolates were found to be negative for methyl red test. All the isolates gave positive reaction for citrate utilization test. All the test isolates showed zone of hydrolysis for starch hydrolysis test. According to the results of performed biochemical tests, the isolates were characterized as *B. subtilis*.

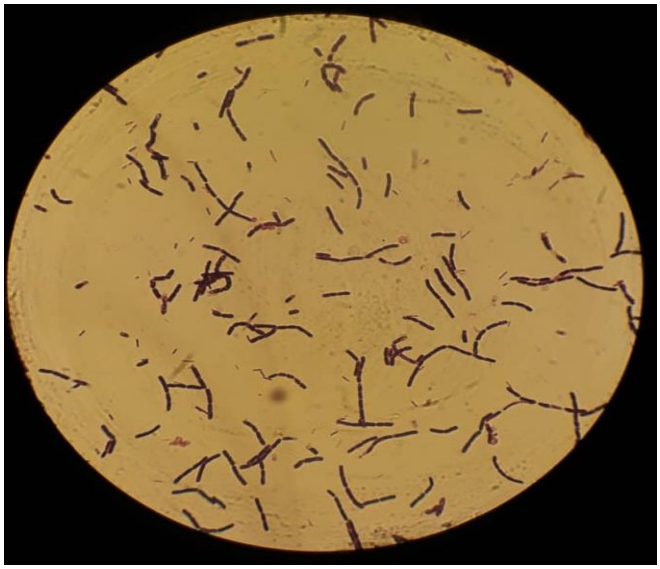

Figure 2. Microscopic view of *B. subtilis* isolates; Gram positive rods were observed under microscope (40X).

***Molecular characterization of B. subtilis isolates*:** Out of total 22 *B. subtilis* isolates, randomly eight isolates {BS1, BS7, BS12, BS19, BS20, BS31, BS35, and BS37 represented as 1, 2, 3, 4, 5, 6, 7, 8 respectively in figure 4.13) were selected for molecular detection based on 16SrDNA sequence. All the isolates showed positive results with an amplicon of 1311bp (Fig.3). This confirmed and differentiated *B. subtilis* isolates from other Bacillus isolates.

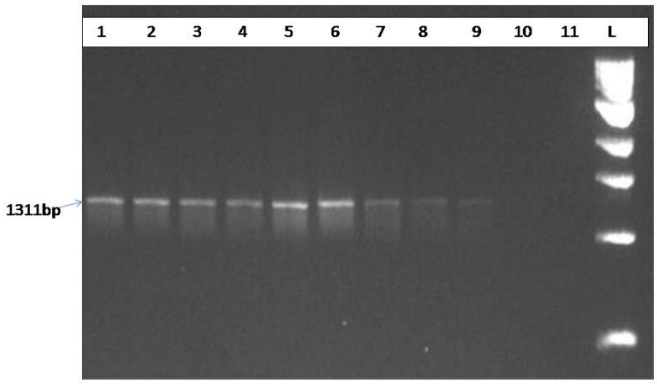

Figure 3. Molecular detection of *B. subtilis* isolates; Lane L represents *DNA* ladder (500bp), lane 1-8 are the isolates, lane 9 is positive control, lane 10-11 are negative controls.

Sequencing and phylogenetic analysis: Out of total eight PCR positive isolates, three (BS7, BS12, and BS31) were selected for sequencing analysis. The sequence data obtained were opened with Bio-edit software and exported as FASTA format of nucleotide sequences. The sequence was analyzed by basic local alignment search tool (BLAST) from NCBI database (Fig. 4).

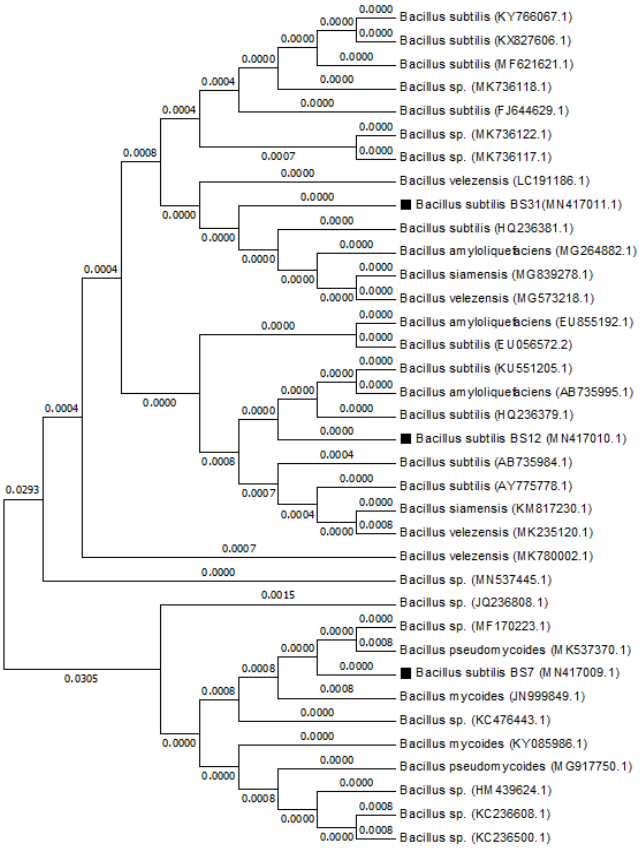

Figure 4. Phylogenetic tree showing relation of *B. subtilis* isolates of *present* study .

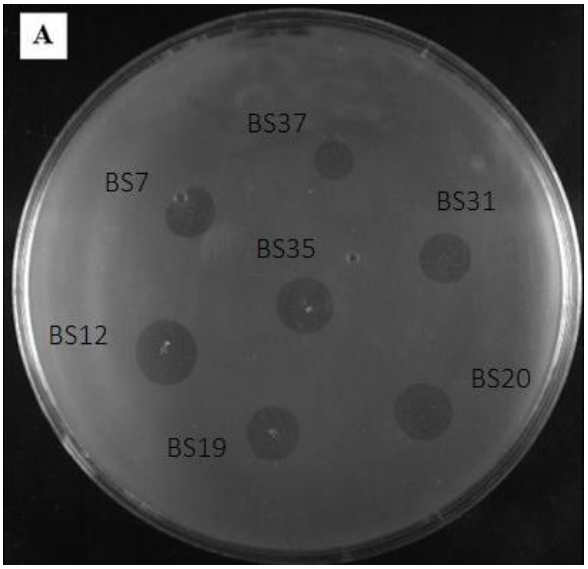

Figure 5. Agar Well Diffusion Assay; inhibition zone formation shows bacteriocin *production.*

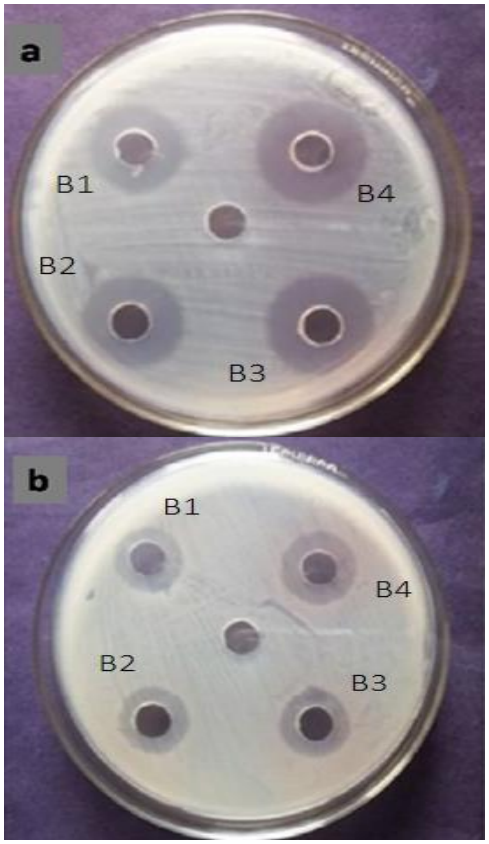

Figure 6. Antibacterial activity of bacteriocin by agar well diffusion assay; (a) inhibition zones shown against *S. aureus*, (b) inhibition zones shown against *E. coli.* In both fig a and b, central well is negative control (broth only) while outside wells contain bacteriocin samples. B1, B2, B3 and B4 are purified bacteriocin samples obtained from samples BS7, BS12, BS21 and BS31.

Screening of bacteriocin producing B. subtilis isolates

***a) Exclusion of inhibitory action due to Lytic Bacteriophages*:** To assess the ability of *B. subtilis* isolates for bacteriocin production, reverse side agar technique was followed. Zones of inhibition were observed for all stabbed cultures which confirmed that the inhibitory activity is due to bacteriocin production (Fig. 7).

b) Exclusion of inhibitory action due to protease production: Clear zones of inhibition were observed around each well. This showed that inhibitory activity was not due to protease production.

***Extraction of bacteriocin and antibacterial activity of bacteriocin*:** Bacteriocin samples were purified from *B. subtilis* isolates; BS7, BS12, BS20, and BS31, were taken and using agar well diffusion assay, antibacterial activity was determined. B1, B2, B3 and B4 are purified bacteriocin samples obtained from samples BS7, BS12, BS21, and BS31. Maximum inhibition zones were observed against *S. aureus* while minimum for *E. coli* (Fig. 8, Table 2).

Table 2. Zone of inhibition observed in antibacterial activity of bacteriocin

Test organism	Zone of inhibition (mm)
E. coli	17+_0.8
S. aureus	21+_0.8

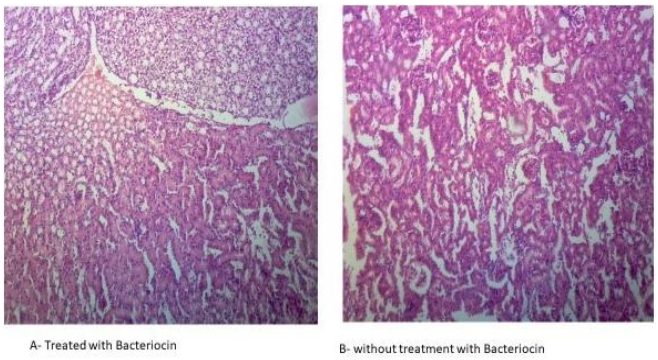

Figure 7. Histopathology of mice organs; No histological changes were observed in tissues of Liver hepatic cords are straight, Portal triad showing no changes.

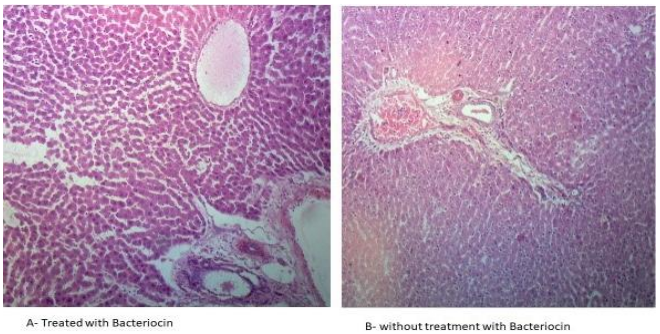

Figure 8. Histopathology of mice organs; H and E-stained sections of Kidney tissue. Tubules are intact there is no degenerative and inflammatory changes observed and no hemorrhages are detected.

Table 3. Bacteriocin purification by Ammonium Sulfate Precipitation method

Bacteriocin sample				After precipitation with Ammonium Sulfate				Purification fold	Product yield (%)
Vol.	**Total protein**	**Total activity**	**Specific activity**	**Vol.**	**Total protein**	**Total activity**	**Specific activity**		
300ml	1.8mg	4800AU	2666.66AU/mg	3ml	0.49mg	1920 AU	3918.36 AU/mg	1.46	40%

***Ammonium sulfate precipitation method*:** Bacteriocin partially purified by ammonium sulfate precipitation method was collected in Eppendorf tubes and dialysate after dialysis was estimated. The results indicated 40% yield of bacteriocin out of total protein (Table 3).

***Lethal Dose 50 (LD_{50}) of Bacteriocin*:** The results indicated that a dose of bacteriocin not greater than 1.0 g/ml did not cause any death of mice up to 14 days of observation. These results highlight bacteriocin importance as safe powder to be used in food.

Table 4. Number of dead and live mice during experiment.

Doses (g/ml)	Dead mice	Live mice
0.25	0	5
0.50	0	5
1.00	0	5

Table 5. Hematological analysis of blood sample taken from experimental and control group.

Parameters	Experimental group 1	Experimental group 2	Experimental group 3	Experimental group 4 (positive control)	Experimental group 5
RBCs	3.67× 10^{12}/l	3.97× 10^{12}/l	3.87× 10^{12}/l	3.50× 10^{12}/l	3.77×10^{12}/l
MCV	49.7fl	61.7fl	59.7fl	75.0	57.7fl
RDW%	17.0	16.0	15.0	11.0	10.0
HCT%	18.2	24.2	19.2	35.0	18.0
PLT	68FD×10^9/l	66FD×10^9/l	65FD×10^9/l	100	66FD×10^9/l
MPV	8.2fl	8.4fl	8.0fl	8.0	8.1fl
WBC	1.2DE×10^9/l	1.9DE×10^9/l	1.4DE×10^9/l	3.5	1.3DE×10^9/l
HGB	5.1g/dl	7.9g/dl	6.1g/dl	11.5	5.5g/dl
MCH	13.9pg	20.0pg	16.9pg	25.0	15.9pg
MCHC	27.9g/dl	32.5g/dl	30.0g/dl	31.0	29.9g/dl
LYM	0.9OM×10^9/l	0.7OM×10^9/l	0.5OM×10^9/l	0.5	0.8OM×10^9/l
GRAN	0.1OM×10^9/l	0.1OM×10^9/l	1.0OM×10^9/l	1.2	0.9OM×10^9/l
MID	0.2OM×10^9/l	0.1OM×10^9/l	0.1OM×10^9/l	0.1	0.1OM×10^9/l
LYM%	72.7OM	62.7OM	57.7OM	15.0	70.7OM
GRA%	8.8OM	7.8OM	5.8OM	35.0	7.8OM
MID%	18.5OM	15.5OM	14.5OM	2.0	16.5OM

Table 6. Analysis of variance (ANOVA) for hematological analysis of blood samples.

Source of Variation	SS	df	MS	F	P-value
Treatment	40173.55	15.00	2678.24	37.10	0.000*
Error	4620.18	64.00	72.19		
Total	44793.73	79.00			

* = Significant, ** = Highly significant

Table 7. Serum profiling of Rabbit blood samples.

Parameters	Exp. G1	Exp. G2	Exp.G3	Exp. G4	Exp.G5
ALT (U/L)	79.00	102.00	87.00	80.00	90.00
AST (U/L)	56.00	75.00	65.00	59.00	70.00
Total protein (d/l)	7.02	7.74	7.49	7.00	7.59
Albumin (d/l)	4.64	4.67	4.68	4.65	4.60
Globulin (d/l)	2.38	3.07	2.81	3.38	3.50
Urea (mg/dl)	79.30	87.10	57.30	89.30	60.30
Creatinine (mg/dl)	1.20	1.10	1.10	1.20	1.10

Table 8. Analysis of variance (ANOVA) of Rabbit blood samples obtained after serum profiling.

Source of Variation	SS	df	MS	F	P-value
Parameters	45464.05	6.00	7577.34	150.49	0.00*
Groups	278.24	4.00	69.56	1.38	0.27
Error	1208.44	24.00	50.35		
Total	46950.73	34.00			

* = Significant, ** = Highly significant, In the ANOVA table, the p-esteem (0.00) for parameters (0.27) for groups that there is adequate proof that not every one of the methods are equivalent when alpha is set at 0.05.

Chemotherapeutic characteristics of bacteriocin

***Results of superficial skin model*:** The chemical property of bacteriocin to treat damaged skin was analyzed. It was found that the damaged skin area showed recovery signs after administration of bacteriocin. Even after 16 hrs of bacteriocin injection, injury started to heal. There was no mortality in treated and controlled group animals during experimentation.

Results of experimental Gram negative septicemia model: In this experiment, different parameters related with immunogenicity of bacteriocin etc. were checked. No signs and symptoms and deaths were observed in groups treated with bacteriocin. The hematological analysis indicated that level of different parameters was almost close to the value of control group. Level of Lymphocytes (LYM) was significantly increased as compared to the control group while the value of Granulocytes (GRA) was decreased. No toxic effect of bacteriocin was observed. There was no increase in body weight and spleen size. No toxic indices like vomiting, depression diarrhea etc. was observed.

DISCUSSION

Bacteriocins have ability to inhibit the growth of other pathogens and thus can be used in food preservation. Bacteriocins are the group of antimicrobial peptides that are produced by many microorganisms. *Bacillus* species are examined good producers of antimicrobial substances, including peptide and lipopeptide antibiotics, and bacteriocins. The ability of Bacillus to produce such antimicrobial substances along with its sporulation capacity confers its survival in different habitats. The following study was planned to produce bacteriocin by *B. subtilis* isolates and also to check therapeutic potential of bacteriocin.

In the present study, *B. subtilis* was isolated from soil samples and confirmed through Gram staining and biochemical characterization. The results indicated that out of total 50 collected samples, 22 were positive for *B. subtilis* showing fuzzy white and slightly yellow color colonies on nutrient agar plates. The pure isolates were Gram positive rods in different arrangements and positive for VP and starch hydrolysis test. Sugar fermentation tests: glucose and manitol fermentation, were also found positive for *B. subtilis* isolates. Similar work was performed by others (Bischoff *et al.,* 1993; Joseph *et al.,* 2013).

Molecular identification of microbes is becoming a popular technique now a day. For phylogenetic and taxonomic studies of many bacteria, the targeting of 16SrDNA gene is now becoming quite common because of it is ubiquitous in all bacteria and its gene sequence is highly conserved. In present research, results of molecular characterization of *B. subtilis* isolates indicated a band of 1311bp. Based on the sequencing results generated after the amplification of the 16S rDNA given in the Results section, the three isolates were confirmed to be *B. subtilis* strains(sequences submitted to NCBI) of this study. Similar findings were also reported by others. Ashe *et al.* (2014) characterized *B. subtilis* isolates using a set of primers. Sorokulova *et al.,* 2008 identified *B. subtilis* species using 16SrDNA and reported 99.8% similarity index.

B. subtilis species have potential to produce bacteriocin protein which can be extracted and purified for later use. In the present research work, the pure isolates of *B. subtilis* were screened for bacteriocin production. A maximum amount of bacteriocin was obtained through ammonium sulfate precipitation method. The concentration of bacteriocin obtained after ammonium sulphate precipitation was 0.49 mg/ 3 ml and the purification fold was 1.46% (Table 3). Inhibition zones were observed against *S. aureus* and *E. coli* bacteria. Khochamit *et al.,* 2015 also used ammonium sulfate precipitation method for bacteriocin purification and found 4% product yield of bacteriocin out of total protein contents. Luo *et al.,* 2008 evaluated antibacterial activity of subtilin bacteriocin and found similar results.

In present study, Lethal Dose 50 (LD_{50}) of bacteriocin was also determined and it was found that at dose 1.0 g/ml no toxic effect was observed. The same value was also reported by others while studying others probiotic safety. *et al.,* 2016 evaluated toxicity effect of pediocin N6 bacteriocin in mice and reported that bacteriocin up to dose of 20mg/l is safer to use without any toxic effect. Botham (2004) also reported the similar study. Another class of bacteriocin called nisin has also been assessed for toxicity effect through oral administration to experimental animals and similar results were reported. The present study indicated that bacteriocin powder has inhibitory effect against Gram negative bacteria with no side effect and help in rapid healing of skin injures. The bacteriocin can modulate immune system by activating the neutrophils and peripheral blood monocytes which trap and kill the bacteria. By activating these cells, bacteriocin indirectly enhance the immune system. Hematological indexes were measured in this study, but No difference hematological indexes were measured in blood from control and treated animals. The values of granulocytes and lymphocytes remain the same in control and experimental groups. No changes in organs and tissues of control and treated groups were observed during histopathological study. Sorokulova *et al.,* 2008 also did toxicity test on probiotics of Bacillus species and reported the similar findings. Marlida *et al.,* (2016) has also performed the similar work using mice as experimental animals.

***Conclusions*:** In conclusion, bacteriocin produced by *B. subtilis* can be used in placement of antibiotics as it can inhibit the growth of bacteria. *B. subtilis* can be exploited in industry for the production of antimicrobial peptides on large scale as therapeutic agents. But before approval of use for large scale consumption, further extensive and toxicological tests should be performed.

***Conflict of interest*:** The authors declare that they have no conflict of interest in publishing this article.

***Statement of ethics*:** This following study has been conducted in accordance with rules and regulations of Institutional Biosafety and Bioethics Committee (IBC) and written informed consent was obtained from the IBC through Office of Research Innovation and Commercialization (ORIC), UAF.

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