PRODUCTION OPTIMIZATION AND CHARACTERIZATION OF BIOACTIVE COMPOUND AGAINST *SALMONELLA* FROM *BACILLUS SUBTILIS* KBB ISOLATED FROM NEPAL

Dwij Raj Bhatta* and **B.P.Kapadnis****
*Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.
**Department of Microbiology, University of Pune, Pune , India.

Abstract: The present study aimed to isolate bioactive *Bacillus* spp. against multidrug resistant *Salmonella* and many other gram-negative bacterial pathogens. Altogether five bioactive *Bacillus* were isolated from soil samples of Nepal and identified as *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus sp.* and *Bacillus cereus* respectively by conventional techniques and DNA sequencing of the 16S rRNA gene. In all five isolates, the isolate *Bacillus subtillis* KBB was most potent antagonist of multidrug resistant *Salmonella* as tested by agar disc diffusion method. The bioactive compound production was optimized and product was purified by TLC, and bioactive molecule was characterized UV, IR NMR and GCMS and identified as peptide compound.

Key words: *Bacillus*; Bioactivity; Production optimization; Peptide compounds; One step solvent extraction; Antibacterial spectrum.

INTRODUCTION

Emergence of multidrug resistance among bacterial pathogens of hospital environment, domestic and industrial environment and in biofilms is reported globally. Hence, effective treatment systems using conventional antibiotics are failed. Since, the pace of new drug discovery is slower than the rate of emergence of resistance, consequently once easily treated infections are now becoming fatal and untreatable. (Maillard 2002; Stickler 2002; Gilbert and McBain 2003; Braoudaki and Hilton 2004). Therefore, screening of potent antibiotic producing microorganisms from the nature and development of novel, broad-spectrum antibiotics specifically targeting the individual bacterial virulence factors is required as for alternative strategies of antimicrobial therapy (Chopra *et al.* 2002). *Bacillus subtilis* is known to produce many antibiotics previously and is generally regarded as safe (GRAS) organisms (Zheng and Slavic 1999; Schallmey *et al.* 2004; Stein 2005). The antimicrobial compounds of *Bacillus subtilis* isolated by ammonium sulphate precipitation from culture broth showed antimicrobial activity against various foodborne pathogens such as *Bacillus cereus*, *Listeria monocytogen and Salmonella* Typhimurium. However, there are no specific reports available on the anti-*Salmonella* activities of *Bacillus* spp. (Bizani *et al.* 2005). Therefore, in present study, an attempt was made for isolation of novel *Bacillus subtilis* strain and also for production, extraction and characterization of partially purified anti-salmonella compound from the isolate.

MATERIALS AND METHODS

Isolation and screening of bioactive *Bacillus* from soil samples

Standard bacteriological media (Hi-media, Mumbai, India) and other chemicals of analytical grade were used in the present study. *Bacillus* spp. were isolated from 10 soil samples randomly collected in the sterile polypropylene bags from cultivated and barren lands in and around Kathmandu by spread plate technique (Collins and Lyne 1989; Priest and Grigoriva 1990). The antimicrobial activity of *Bacillus* isolate was checked by cross streaking technique. The overnight growth of each target culture viz; *Salmonella* Typhi, *E. coli*, *Staphylococcus aureus* was streaked across the growth of *Bacillus* isolates on NA plate and incubated for 24 h at 37°C. Absence of growth adjacent to *Bacillus* growth indicated inhibition of target culture. The bioactivity of the *Bacillus* isolates was further tested by modified agar disc diffusion technique using Bacillus lawn Agar discs against target cultures viz; *Salmonella* Typhi, *E. coli* and *Staphylococcus aureus.* The *Bacillus* isolates that showed broad spectrum and bigger inhibition Zone (IZ) against representative target cultures of *Salmonella* were assumed to be promising bioactive *Bacillus* isolates.

Author for Correspondence: Dwij Raj Bhatta, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal, Email: nidwija@wlink.com.np.

Identification of *Bacillus* isolates by conventional method and by 16S rRNA gene sequence analysis:

Promising *Bacillus* isolates were characterized using the tests prescribed in Bergey's Manual of Systematic Bacteriology (Berkley *et al.* 1984; Claus and Berkley 1986; and Collins and Lyne 1989). All *Bacillus* isolates were identified to species level by 16S rRNA gene sequence analysis using the taxonomy approach (Stackebrandt and Goebel, 1994).The nucleotide sequence analysis of the 16S rDNA of the isolates was done at NCBI server using BLAST-n (www.ncbi.ncm.gov/blast) by aligning the partial sequences obtained with the primers mentioned above. Similarity search for the nucleotide sequence of 16S rDNA of the test isolates done online at the www.ncbi.nlm.nih.gov using BLAST search programme with default parameter for the nucleotide database of Genbank, revealed the tentative identification of the isolates.

Antibacterial spectrum of culture supernatant of promising bioactive *Bacillus*

The primarily screened bioactive *Bacillus subtilis* KBB isolate was then inoculated into 100 ml LB broth and incubated for 24 h at 37°C. After incubation, 50 ml of culture broth was transferred to another 100 ml of sterile LB broth in a 250 ml conical flask, and incubated for 48 h at 37ÚC in the incubator shaker (150 rpm). An aliquot (10 ml) of culture broth was withdrawn from flask after 36hrs and centrifuged at 10,000 rpm for 15 min. The supernatant was filtered through 0.22 ìm Millipore membrane filter (Sartorius, Germany). An aliquot (5 µl) of each culture supernatant was spotted onto MHA plate spread inoculated with overnight growth of target cultures. After incubation at 37°C for 18-24 h, the plates were observed for inhibition of target cultures.

Selection of medium of antimicrobial production

The *Bacillus subtilis* KBB was inoculated into 500 ml LB broth (pH 7.0) in 1000 ml flask and incubated for 24 h at 45°C. After incubation, 50ml culture was inoculated to 250 ml conical flasks each containing 100 ml Nutrient broth, Mueller Hinton broth, LB broth, and LB broth supplemented with additional carbon source (1% glucose, sucrose, and maltose) along with LB broth medium containing 1% maltose, 0.1% $MnCl_2$ and 0.1% KNO_3 (pH 6. 8). All flasks were incubated at 37°C in shaking condition (150 rpm) for 36 h. After 36 h cell free supernatants were obtained from all flasks by centrifugation at 15000rpm for 15 min. The antimicrobial activity of all cell free supernatants was determined against *Salmonella* Typhi W7 by spot on lawn method.

The time course of antimicrobial compound production

The time course of antimicrobial compound production by *B. subtilis* KBB was studied in LB broth with 1 % Maltose, 0.1% $MnCl_2$ and 1% KNO_3 (Bacillus broth) (pH 6.8) for 5 days against target organism *Salmonella* Typhi.

Bacillus subtilis KBB (2 ml, 10^6 cells/ml) was inoculated into 200 ml Bacillus broth (pH 6.8) and incubated in shaker incubator (150 rpm) for 5 days at 37°C. At every 12 h interval, 10ml of culture was withdrawn from the flask. The growth was determined in terms of OD at 600nm. Part of the culture suspension was centrifuged (15000 rpm) and the supernatant was filtered through 0.22 µm pore size membrane filter (Sartorius, Germany). The supernatant was assayed for protease activity as well as antimicrobial activity against *Salmonella* Typhi by spot on lawn method (Korenblum *et al.* 2005).

Assay of antimicrobial activity in terms of Arbitrary units (AU)

Antimicrobial activity was determined in terms of Arbitrary units (AU) which is defined as the reciprocal of the highest dilution of the supernatant that inhibited target organism x 1000, divided by the volume of supernatant applied on the spot. Two-fold dilutions of the supernatant were made in sterile distilled water. 5 µl of each dilution spotted on MHA plates (pH 7.2) seeded with *Salmonella* Typhi W7 (10^6 cells/ml). These plates were incubated at 37°C for 24 h (Korenblum *et al.* 2005). The increase in the antimicrobial activity was detected up to 3rd day of fermentation (36 h) and slight decrease thereafter. It was thought that the host protease production might have resulted in the decrease of activity. Therefore, along with time course of production the protease activity was also assayed by Anson modified method (Anson 1939).

Determination of temperature stability of culture supernatant bioactivity

The effect of temperature, pH, enzymes and solvents on the stability of bioactive compounds in culture supernatant (filter sterilized) of *Bacillus* KBB by the method of Korenblum *et al.* (2005). Each assay was performed three times in duplicate (Teo and Tan 2005).

Extraction of bioactive principle from culture supernatant of* Bacillus subtilis *KBB

In present study the antimicrobial compound was extracted by one step solvent extraction procedure by modification of Bligh and Dyer method 1959 (www.cyberlipid.org). The culture supernatant was mixed with equal volume of chloroform and agitated vigorously in a separating funnel. Absolute methanol (4 times the culture supernatant) was added slowly to it. The aggregate / precipitate observed at the interfacial region was collected in a petridish and evaporated. The residue was termed as crude antimicrobial compound (CAC). The residue (2 mg) was dissolved in 500 µl of distilled water and its activity was checked by spot on lawn method against *Salmonella* Typhi .

Partial purification of extracted antimicrobial substance

From 100 ml broth 200 mg of CAC (dry residue) was obtained. This residue was distributed into 2 portions (100mg) in 500 µl of absolute ethanol in sterile eppendorf tubes and incubated for 1 h. Then the tubes were centrifuged and the ethanol layer was separated. The residue was kept in the incubator at 45°C to evaporate remaining ethanol. The residue from both the tubes was dissolved in 500 µl of sterile double distilled water, mixed togather, and extracted again with methanol chloroform method as described under 5.2.8 ii. The aggregate / precipitate from the interfacial region was collected in

petridish and dried at 45°C in the incubator. The resulting residue (100mg) was distributed into small portions in sterile fresh set of eppendorf tubes. In both tubes 500 µl of absolute ethanol was added and kept for 1 h. Then the tubes were centrifuged and the residue from the tubes was dried at 45°C. On re-dissolving the residue in 500 µl of double distilled sterile water, a yellow coloured solution obtained to which 2 ml of absolute methanol was added slowly. The aggregate / precipitate was observed and was kept in incubator at 45°C to evaporate methanol and water. The residue obtained was suspended in 500 µl of absolute ethanol in eppendorf tube and ethanol was evaporated. The residue obtained was termed as partially purified antimicrobial compound. The partially purified antimicrobial compound was tested for purity by TLC using pre-coats (Polygram® Sil G/UV 254,Macherey-Nagel) and methanol: water: chloroform (10:20:5) as solvent system.

Characterization of antimicrobial compound

Biuret and Ninhydrin tests were performed to confirm protein nature of partially purified compound (Plummer 1997). Protein in the extract was estimated by Folin Lowery method using BSA as a standard (Plummer 1997).

Proteins were analysed in 14% SDSPAGE by Laemmli method (1970). An aliquot (10 µl) of partially purified antimicrobial compound (protein concentration 100 µg per ml) was treated with an equal volume of SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 2% bromophenol blue) and electrophoresed at 100 V at 28°C for 90 min. The gel was stained with 0.25% coomassie brilliant blue in methanol-acetic acid-water (40: 10: 50), and destained in the same solvent to observe the protein bands. A comparison was made with mobility of SDSPAGE standards (Sigma) (Korenblum *et al*. 2005; Bizani *et al.* 2005).

Determination of purity and activity of antimicrobial compound by TLC and **bioautography**

An aliquot (5 µl) of partially purified antimicrobial compound (water solution) was spotted on 2 TLC precoats (Polygram® Sil G/UV 254,Macherey-Nagel) and separated with methanol: water: chloroform (10:20:5) solvent system. The original concentration of each extract in this experiment was 100 mg/ml. One of the dried plates was kept on petriplate and overlaid with MHA seeded with *Salmonella* Typhi W7 and incubated at 37°C for 18 h and observed for inhibition. Second plate was developed with Ninhydrin. The distance moved by active compound and solvent was recorded and the Rf value of active compound determined (Plummer 1997). In this experiment the position of active compound (as indicated by inhibition Zone) on overlaid TLC plate was located and compared with that detected by Ninhydrin.

Determination of antimicrobial Spectrum of partially purified compound

The partially purified antimicrobial compound was dissolved in double distilled water (100 mg/ml) and 5 µl of it was spotted on the lawn of target cultures (multi drug resistant *Salmonella* isolates). The plates were incubated for 24 h at 37ÚC. After incubation inhibition zones were recorded (Korenblum *et al*. 2005).

Determination of minimum inhibitory concentration (MIC) of partially purified compound

MIC of partially purified antimicrobial compound against *S.* Typhi and *Staphylococcus aureus* was determined by broth dilution technique. Briefly, the partially purified antimicrobial compound was dissolved in double distilled water (100mg/ml) and double dilution of the compound was made in nutrient broth. Then, 1 ml inoculum (10^6 cfu ml^{-1}) of *Salmonella* Typhi and *Staphylococcus aureus* was added separately to an equal volume of two-fold dilutions of respective antibicrobial solution. For positive control, 1 ml inoculum (10^6 cfu ml^{-1}) of target organisms was added to 1 ml growth medium without antimicrobial. All the tubes were incubated for 24 h at 37°C. The MIC was reported as the lowest concentration of antimicrobial substance that prevented visible growth (Cheesbrough 1993; Andrews 2001).

UV, IR, GCMS and NMR analysis of bioactive molecule

The UV absorption spectrum analysis was done using double beam UV spectrophotometer (UV 1601, Shimadzu Japan). The FTIR (Fourier Transform Infrared) spectrum was conducted using FTIR 8400 (Shimadzu, Japan). GC-Mass spectroscopy was conducted (GCMS QP5050, Shimadzu, Japan) to characterize bioactive compound. In present study ^{1}H (proton, i.e., PNMR) and ^{13}C (carbon- 13, i.e., CNMR) NMR spectroscopy (Varion mercury YH 300) was done to analyse the spectral data. Above-mentioned analysis was conducted at Garware Centre, Department of Chemistry, University of Pune, India, according to standard protocol.

RESULTS

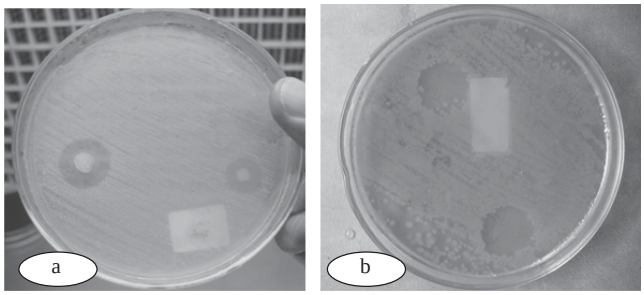
Antimicrobial spectrum Bioactive *Bacillus* isolates and their identification

Altogether five bioactive Bacillus isolates viz; KBR, KBB, KBY, KBC and KBA, showed activity against *E. coli*, *S.* Typhi and *Staphylococcus aureus* in primary screening by cross-streaking method. Their bioactivity was subsequently confirmed by agar disc diffusion method. In all five bioactive isolates isolate KBB was found to be more potent antagonist of multidrug resistant *Salmonella* as tested by agar disc diffusion method (Plate 1).

It was identified as *Bacillus* spp. on the basis of morphological and biochemical characteristics by conventional techniques (Berkley *et al.* 1984; Claus and Berkeley 1986 (Plate 2).

The partial sequence of the 16S rRNA gene of the strain KBB was also obtained with different primers which showed closest homology with some known sequences of *Bacillus* sp. in Gene bank after first five blast hits (On NCBI BLAST). Therefore, the strain was tentatively identified as *Bacillus subtillis* and the partial sequence was submitted to NCBI gene bank with accession number EF42850 (Fig 1).

Plate 1: Bioactivity of *Bacillus* subtilis KBB against *Salmonella* Typhi W7



a. Agar disc method, b. Spot on lawn method

Plate 2: Photomicrograph of *Bacillus subtilis* KBB

Strain KBB

Primer used for sequencing **343R**

Homology with *Bacillus subtilis* 98%

Sequence is submitted to Gene bank with accession numberEF428450

TCAGTGTGGCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTGAGCCGTTACTC
ACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCCGAAGCCACCTTTTATG
TTTGAACCATGCGGTTCAAACAACCATCCGGTATTAGCCCCGGTTTCCCGCCACTTATCCCAGT
CTTACAGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAACATCAGGGA
GCAAGCTCCCATCTGTCCGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCGTCCT
GAGCCATGATCAAACTCTGGAGCTGCT

First five hits

gi|29164925|gb|AY219900.1|	Bacillus subtilis 16S ribosomal RNA g	565	2e-158
gi|93210287|gb|DQ462193.1|	Bacillus subtilis strain MP-3 16S ...	559	1e-156
gi|71493066|gb|DQ122328.1|	Bacillus sp. iCTE54 16S ribosomal ...	559	1e-156
gi|71493065|gb|DQ122327.1|	Bacillus sp. iCTE51 16S ribosomal ...	559	1e-156
gi|71493064|gb|DQ122326.1|	Bacillus sp. iCTE50 16S ribosomal ...	559	1e-156

Strain KBB

Primer used for sequencing 530F

Homology with *Bacillus subtilis* 99%

CGATGTCGGATATTGGGCGTAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCC
CCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGA
GAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACC
AGTCGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGT
GGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAG
TGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTAC
GGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGAAGCAACGCGAAGAACCT

gi|59859126|gb|AY913755.1|	Bacillus subtilis strain CICC10078...	825	0.0
gi|58615438|gb|AY881646.1|	Bacillus subtilis strain CICC10074...	825	0.0
gi|58615435|gb|AY881643.1|	Bacillus subtilis strain CICC10048...	825	0.0
gi|58615428|gb|AY881636.1|	Bacillus subtilis strain CICC10026...	825	0.0

***Strain* KBB**

Primer used for sequencing 704F

Homology with *Bacillus subtillis* 100%

CCGTGGCGAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGC
GAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGC
CCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGCCGCAAGACTGAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTA
CCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACA
GGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGAC
TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCT
TATGACCTGGGCTACACACGTGCTACAATGG

First five hits

gi|48596227|gb|AY590138.1|	Bacillus sp. SXQ-2004 16S ribosomal R	1019	0.0
gi|47118164|gb|AY601724.1|	Bacillus sp. AMX-4 16S ribosomal RNA	1011	0.0
gi|47118163|gb|AY601723.1|	Bacillus sp. WL-3 16S ribosomal RNA g	1011	0.0
gi|47118162|gb|AY601722.1|	Bacillus subtilis isolate WL-7 16S...	1011	0.0
gi|93211252|gb|DQ474759.1|	Bacillus subtilis strain E9 16S ri...	1011	0.0

Figure 1: 16S rRNA gene sequencing of bioactive Bacillus subtillis KBB.

Time course of antimicrobial activity from *Bacillus subtilis* KBB

Bigger Zone of inhibition of *Salmonella* Typhi was observed by the cell free culture supernatant of *B. subtilis* grown in LB broth supplemented with 1% maltose, 0.1% $MnCl_2$ and 0.1% KNO_3 (pH 6. 8) then by the culture supernatant of Nutrient broth, Mueller Hinton broth, LB broth, LB broth supplemented with carbon source (1% glucose, sucrose, and maltose). This medium was designated as Bacillus broth, which enhanced the production of anti-Salmonella compounds. Time course of antimicrobial compound production by *B. subtilis* KBB in Bacillus broth was monitored over a period of 5 days. Maximum activity was observed on the 3rd day. The rate of antimicrobial compound production correlated with growth rate of *Bacillus subtilis* KBB and was highest (1600 AU/ml) in the late log phase. During the course of antimicrobial compound production, initial pH of the fermentation broth on first day was neutral and was raised to 7.5 after 12 h and 8.2 after 24 h and it remained constant 24 h onwards. During the course of antimicrobial compound production the protease activity was negligible (0.27 unit/ml) and remained same upto 96 h. It was same even when there was maximum production of antibiotics at 36 h. It indicates that the organism did not produce significant amount of protease and therefore, no possibility of loss of activity.

Table 1: Time course of antmicrobial activity of Culture Supernatant of *Bacillus subtilis* KBB

Incubation (h)	Growth (OD_{600})	Activity (AU/ml)	Zone of inhibition+ (mm)	pH of CS	Protease (Units/ml)
0	0.223	0	NZ	6.8	
12	1.113	200	5	7.5	ND
24	1.773	400	15	8.2	0.27
36	1.995	1600	16	8.2	ND
48	1.888	1600	18	8.2	0.27
72	1.231	800	15	8.2	0.27
96	0.914	400	12	8.2	0.23

+Against *S.*TyphiW7

Stability of antimicrobial compound at different temperatures

When the antimicrobial activity of the stored culture supernatant (stored for one month at -20°C, 4°C, and at 28°C) was determined against *Salmonella* Typhi, the activity was similar to that of control (before storage). It was concluded that the antimicrobial compound did not lose any activity even after one month storage in refrigerator and at 28°C, which indicates that the compound do not undergo any kind of degradation during storage at varied temperature.

Effect of temperature, pH, enzymes, and solvents on bioactivity of culture supernatant of *Bacillus subtilis* KBB

The antibacterial activity was not affected until 90°C and then decreased slightly thereafter and lost completely after autoclaving. These results indicated that the antimicrobial compound seems to be highly thermostable upto 90°C **.**

The initial pH of the culture supernatant was 8.2 and was adjusted to 2.5, 3.8, 4.2,5.2,5.8,6.8 with citrate phosphate buffer and raised to pH 9.8, 10.2 by carbonate-bicarbonate buffer. Antimicrobial activity was observed within wide pH range. However, the activity was affected at extreme pH .

The culture supernatant of the *B. subtilis* KBB was treated with enzymes as described in methodology. After the enzyme treatment the supernatant was tested for antimicrobial activity. The inhibition zone diameter produced by supernatant treated with proteinase K, pronase and ∝ chymotrypsin, was smaller than that of untreated sample. It indicates that the antimicrobial compound is partially susceptible to proteinase K, pronase and ∝ chymotrypsin and insensitive to pepsin. There is no loss of antimicrobial activity observed with ethyl alcohol, methanol, acetone, and Chloroform.

Solvent extraction of antimicrobial compound

In the present study, 100 mg of dry residue was obtained from 100 ml (1 g/l) of cell free supernatant and termed as crude antimicrobial compound (CAC). The CAC was completely soluble in distilled water, giving yellow coloured solution. The pH of the solution was acidic (pH 3.5). Subsequently, 2 mg of CAC residue was dissolved separately in 500 µl of distilled water, and it was highly bioactive active against *Salmonella* Typhi. The residue was insoluble in acetone, chloroform, ethanol and methanol. In acetone in a Petridis the residue formed sticky and hard structure like calcification. In chloroform also it was completely insoluble. However, it was soluble in 25% methanol and ethanol.

The partial purification of the compound by successive solvent washing resulted in 50 mg of dry residue from 100 ml of culture supernatant. Finally obtained residue was termed as partially purified antimicrobial compound. The partially purified residue was also soluble in water but not in acetone, chloroform, and absolute methanol and ethanol. The aqueous solution was highly acidic in nature in water solution, and yellow coloured with characteristic smell. The solution was distributed in eppendorf tubes in small quantity. Some were kept at 28 °C and few were kept at 5°C and tested for stability. The compound was found bioactive even after one-year storage at 28°C. The partially purified antimicrobial compound was tested for purity by thin layer chromatography (Polygram® Sil G/UV 254,Macherey-Nagel) and only one spot observed with Rf value 0.5 confirmed the purity of the compound.

Table 2: Antimicrobial spectrum of partially purified compound from *Bacillus subtilis* KBB

TARGET CULTURES			Zone of inhibition with anticrobial compound**
Isolate	Organism	Antibiotic resistance*	
W1	*S.* Typhi phage type UVS1	AR	++
W7	S. Typhi phage type A	AR	++
N3	S. Typhi phage type E1	MDR	+
N5	*S.* Typhi phage type UVS4	MDR	++
N7	*S.* Typhi phage type E1	MDR	+
B1	*S.* Typhimurium,	MDR	++
B4	*S.* Typhimurium,	MDR	+
B6	*S.* Typhimurium	MDR	+
W2	*S.* Paratyphi A	AR	+
W9	*S.* Paratyphi A	MDR	+
N13	*S.* Enteritidis	MDR	+
D23	*Citro. Fruendii*	ND	++
D15	*Ent. Agglomerans*	ND	++
E13	*E. coli*	MDR	++
D24	*M. morganii*	ND	+
D28	*P. mirabilis*	ND	++
St2	*Staph. Aureus*	ND	+++
-	*Fusarium spp.*	ND	++

** Suceptibility of target cultures to partially purified compound by spot on lawn method (Inhibition zone produced by partially purified compound 100 mg/ml solution and 5µl spot)

+++ inhibition Zone 25 mm; ++ inhibition Zone ≥ 15 mm; + inhibition Zone ≤ 15mm

* Refer chapter 3 Table 3. 3; and chapter 2 Table 2. 8.

AR= ampicillin resistant; MDR= multidrug resistant; ND antibiotic sensitivity not determined.

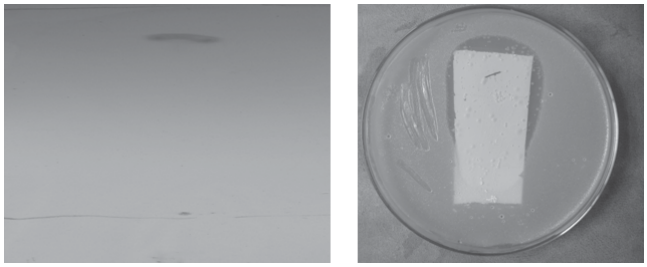
Proteinaceous nature of antimicrobial compound

The property of partially purified compound as studied by Biuret test, and Ninhydrin test confirmed its proteinaceous nature. In TLC plate the compound was detected with Ninhydrin reagent after heating the plates at 100°C for 5 min. Only one spot was observed with 0.5 Rf value confirmed purity and protein nature of extracted antimicrobial compound. The protein concentration in the 100 mg/ml of partially purified compound was 0.87 mg/ml as determined by Folin Lowery method (Plummer, 1997). The compound was resolved in 14 % gel. A single band in SDS PAGE approx. 14 KD confirmed the proteinaceous nature and low molecular weight of the antimicrobial compound.

TLC bioautography

Two thin layer chromatography plates (Polygram® Sil G/UV 254,Macherey-Nagel) of similar size were spotted with solution of partially purified antimicrobial compound at similar distance and fractionated with methanol: water: chloroform (10:20:5) solvent system. The original concentration of each extract in this experiment was 100 mg/ml. A single spot of the compound with Rf value 0.47 observed on TLC plate developed with Ninhydrin confirmed, the presence of single compound in cell free supernatant. The Rf value 0.5 of bioactive spot observed on second TLC plate seeded with target culture was similar to that detected in first plate confirmed the compound was bioactive (Plate 2).

Plate 2: TLC bioautography of antimicrobial compound of *Bacillus subtilis* KBB



(a) (b)

(a) Single spot in TLC visualized with Ninhydrin (b)TLC bioauto graphy

Antimicrobial spectrum of partially purified antimicrobial compound

The spot on lawn method indicated that the partially purified compound was active against many gram negative pathogens viz; MDR *Salmonella, E. coli* etc. and *Staphylococcus aureus*.

Minimum inhibitory concentration of partially purified antimicrobial compound against *Salmonella* Typhi and *S. aureus*

The minimum inhibitory concentration of partially purified antimicrobial compound was found to be 125 ìg/ml for *Staph. aureus* and 250 ìg/ml for *Salmonella* Typhi .

Characterization of antimicrobial compound by UV, IR, GCMS, NMR spectrum analysis

The low molecular weight of the antimicrobial compound with a single band with SDS PAGE and single spot developed with Ninhydrin on TLC plate indicated the purity of the compound. The HPLC peak analysis, UV Spectrum, IR Spectrum, GCMS Spectrum and NMR Spectrum supported the peptide nature of the compound. The UV absorption spectrum (UV 1601, Shimadzu Japan) of the antimicrobial compound was examined between 190 and 600 nm. The compound showed absorption maxima at 205 nm was corresponding to characteristic absorption of peptide bonds. A shoulder at 268 nm indicated the protein nature of the compound. The FTIR (Fourier Transform Infrared) spectrum (FTIR 8400,Simadzu, Japan) exhibited characteristic absorption valley at 1765 Cm^{-1} (carboxyl group), valley at 1664.5 Cm^{-1} (Gausian amide bonds) and valley at 3138 Cm^{-1} (hydrogen bonded OH groups). All indicated that substance contains peptide bonds. C-NH_2 stretching was indicated by valley at 979 cm^{-1} and carbon - carbon stretching was indicted by valley at 1652 cm^{-1}. The valley at 2665 cm^{-1} shows C-H stretching, valley at 619 shows 4-Amino quinaldine or Na acetate or C-C inplane bending, and valley at 14004.1cm^{-1} shows symmetric COO^-stretching. The O-H stretching was indicted by valley at 3138. All above characteristics valleys indicated peptide-based structure of the compound. The GC-Mass spectrum data was analysed (GCMS QP5050, Shimadzu, Japan). The analysis of all major peaks (molecular mass 207) indicated that compound closely resemble to peptide type antibiotics in the antibiotic library. But it also resembled to many antibiotics and mainly to silicon (Si) containing compounds in the library but not exactly. Therefore, the compound must be a new molecule. The ^1H NMR (Nuclear magnetic resonance, 500 MHz) ^{13}CNMR spectra of the antimicrobial compound in D_2O indicated presence of 13 H and 19 C in the sample. However, the complete structure elucidation of the compound and molecular formula could not be established in this study and further study is recommended (fig 2.,fig3,fig4.,fig.5)

Table 3: Physico-chemical properties of the antimicrobial compound from *Bacillus subtilis* KBB.

Properties	Results
Colour	Dark brown when extracted and light yellow after purification
Nature	Amorphous
Yield (mg/lit)	500
Solubility	Water soluble and soluble in 25% methanol, ethanol but insoluble in Absolute methanol and Ethanol and acetone, chloroform
UV λmax (nm)	205
IR (KBr) cm-1	Major valleys 1765 Cm-1 (carboxyl group), valley at 1664.5 Cm-1 (Gausian amide bonds) and valley at 3138 Cm-1 (hydrogen bonded OH groups), indicates peptide nature
GC-MS (m/z)	Probable compound, Peptide

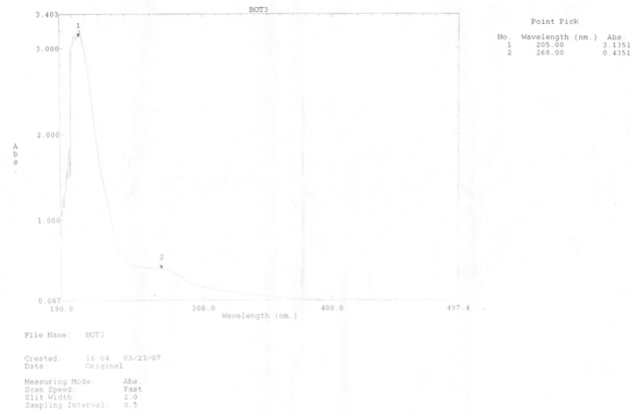


Fig 2: UV Spectra of antimicrobial compound of *Bacillus subtilis* KBB

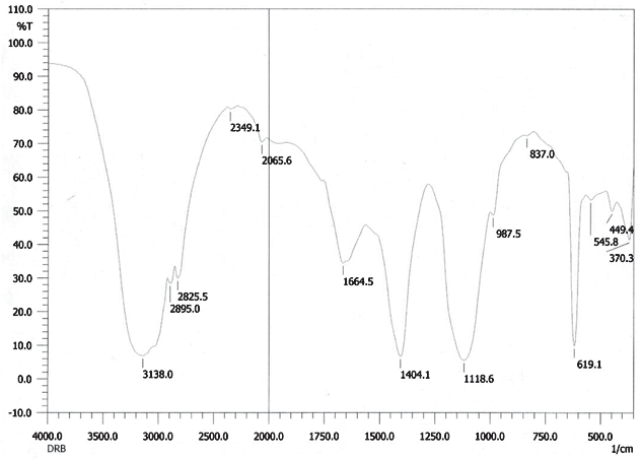
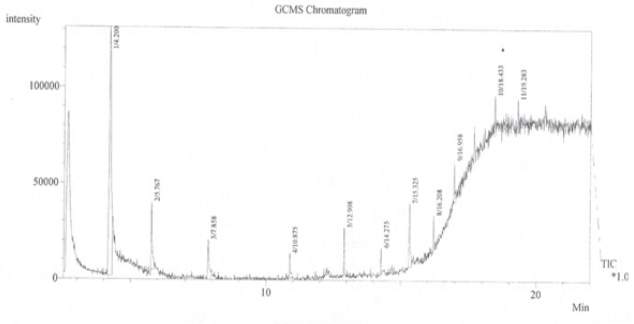


Fig 3: FTIR Spectra of antimicrobial compound from *Bacillus subtilis* KBB

SHIMADZU ANALYTICAL TECHNIQUE CENTRE
DEPARTMENT OF CHEMISTRY
PUNE UNIVERSITY

GCMS Sample Information

Data Acquired by : D. S. Shishupal
Acquisition Date : 6/8/2006 6:04:37 PM
Sample Name : DRB
Sample Amount : 1.000
Injection Volume : 1.000
Data File : C:\GCMSsolution\Data\Project2\DRB1.QGD
Method File : C:\GCMSsolution\Data\Project1\Sanjay SB 154.qgm
Tuning File : C:\GCMSsolution\System\Tune1\A.qgt



GCMS Peak Report

Peak#	R.Time	Area	%Total
1	4.200	672574	57.64
2	5.767	97300	8.34
3	7.858	73882	6.33
4	10.875	34140	2.93
5	12.908	35769	3.07
6	14.275	17422	1.49
7	15.325	95111	8.15
8	16.208	25231	2.16
9	16.958	37908	3.25
10	18.433	41432	3.55
11	19.283	36177	3.10
		1166942	100.00

Fig. 4: GCMS spctra of antimicrobial compound of *Bacillus subtilis* KBB

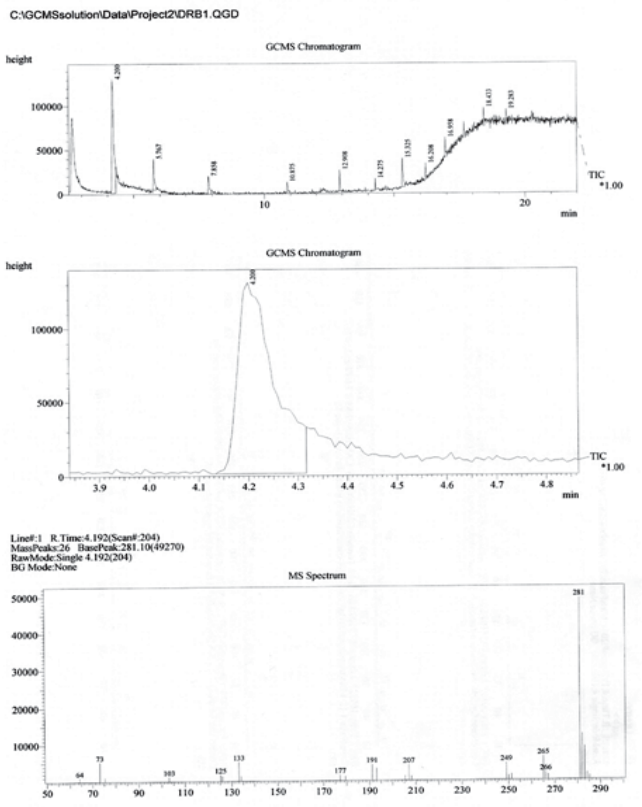
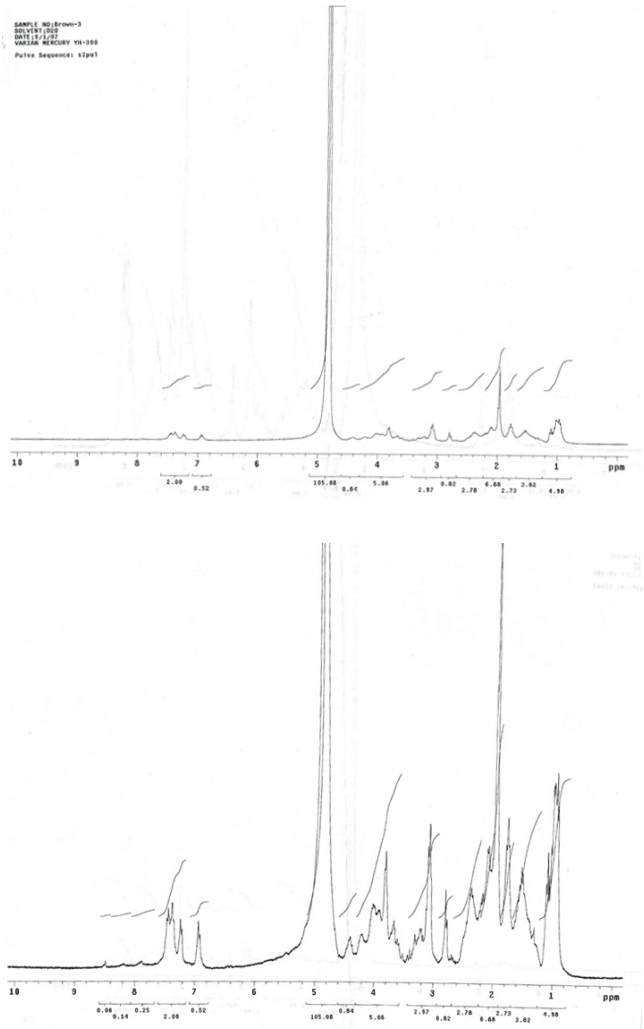


Fig 4: GCMS Spectra of antimicrobial compound of *Bacillus subtilis* KBB



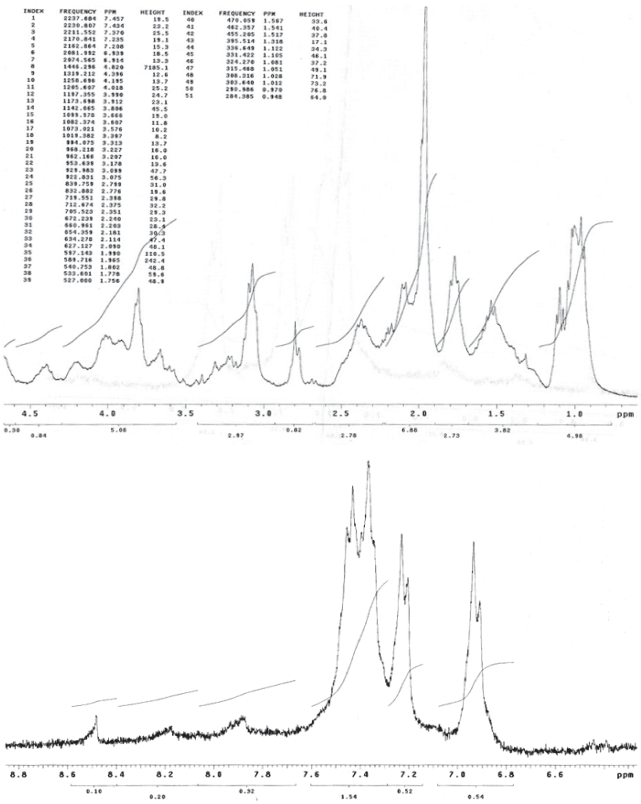
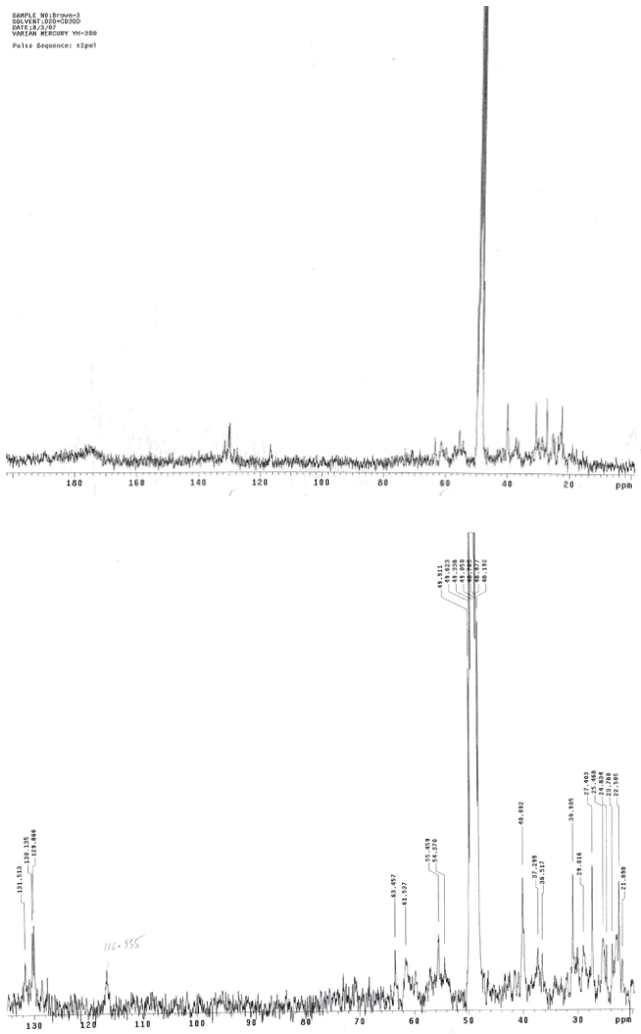


Fig 5: [1]H NMR spectra of antimicrobial compound of *Bacillus subtilis* KBB



```
13C OBSERVE

exp999  std13c

       SAMPLE                    DEC. & VT
date    Mar  7 2007   dfrq            300.063
solvent        D2O    dn                  H1
file           exp    dpwr                37
   ACQUISITION        dof                  0
sfrq         75.458   dm                 yyy
tn             C13    dmm                  w
at           1.815    dmf              10800
np           68106        PROCESSING
sw         18761.7    lb                8.00
fb           10400    wtfile
bs               8    proc                ft
tpwr            59    fn          not used
pw             8.7
d1               0    werr
tof              0    wexp
nt           40000    wbs
ct           33064    wnt
alock            n
gain       not used
      FLAGS
il               n
in               n
dp               y
      DISPLAY
sp           857.7
wp         13332.3
vs            1072
sc               0
wc             240
hzmm         55.55
is          500.00
rfl        5472.1
rfp        3700.8
th              13
ins        100.000
nm  no  ph
```

Fig 6: [13] C NMR spectra of the antimicrobial compound of *Bacillus subtillis* KBB

DISCUSSION

The present study was based on previous studies, which have shown that the *Bacillus subtilis* produces thermo-stable proteinaceous antimicrobial factor, which remains stable in the presence of bile salt and solvents and can be used against animal and human pathogens (Seah *et al.* 2002).

In present study, one of the strain *Bacillus subtilis* KBB was found superior antagonist of many gram negative waterborne pathogens and inhibited the growth of MDR *Salmonella* serovars such as *S.* Typhi, *S.* Typhimurium, *S.* Enteritidis and *S.* Paratyphi A. There are various reports available on production of anti bacterial compound from *Bacillus* viz; lichenin by *B. licheniformis*, bacteriocins called cerein by *Bacillus cereus*, and pumilin production from *Bacillus pumilus* (Katz and Demain 1977; Pattnaik *et al.* 2001; Paik *et al.* 2000). Bacteriocin production by *B. subtilis* has been reported previously, and the best-characterized bacteriocins are subtilin, but reports on anti microbial activity against gram-negative enteric pathogens are scanty and very few reports are available on the broad-spectrum antimicrobial production (Jansen and Hirschmann. 1944; Stein 2005). However, there have been no reports on anti-microbial activity of *B. subtilis* against multidrug resistant serovars of *Salmonella.*

Present study revealed production of low pH anti *Salmonella* compound by *B. subtilis* KBB, which also inhibits *Proteus*

mirabilis, E. coli, Enterobacter agglomerans, Citrobacter frundii and *Morganella morganii*. Therefore, findings have established this strain as the potential broad-spectrum antagonists of waterborne pathogens.

In present study, the antimicrobial compound production from *B. subtilis* KBB was optimized using different liquid media, such as nutrient broth, Mueller Hinton broth, LB broth, and LB broth with additional carbon source (1% glucose, sucrose, and maltose) along with source of manganese. LB broth medium containing 1% maltose and 0.1% $MnCl_2$ and 0.1% KNO_3 was found most suitable for the production of anti-salmonella compound. Manganese has been mentioned as the sporulation inducer and stimulatory agent for antimicrobial compound production (Berkley *et al.* 1984; Peypoux *et al.* 1999; Wei and Chu 2002).

The time course of production of antimicrobial compound indicated that the maximum antimicrobial activity was produced on the 3rd day of fermentation and decreased thereafter. The findings of this study are similar to those reported previously. It has been reported that most of the *Bacillus* antimicrobial compounds are produced by in the late log phase, may be due to sporulation (Katz and Demain 1977). It was speculated initially that decrease in the activity after 3rd day might be due to protease production. Therefore, protease production was also monitored during fermentation cycle.

Since *Bacillus* species are reported to produce peptide antibiotics and proteases as well and it is also possible that protease enzymes catalyze the cleavage of peptide bonds of the protein based antimicrobial compound and makes it inactive. It was believed earlier that a molecule of gramicidin S is stable to proteolytic enzymes due to the presence of two residues of D-phenylalanine in its structure. But various studies have shown that the production of proteinase by *Bacillus subtilis* during antibiotic production is responsible for hydrolysis of polypeptide antibiotic gramicidin S. It has been reported that enzyme gramicidinase formed during sporulation can reduce 45% of gramicidin S after 24 h incubation at 37°C. Similarly, enzyme nisinase produced by *Bacillus polymyxa* is responsible for inactivation of nisin (Jarvis 1967; Katz and Demain 1977; Stein 2005). Unlike previous studies, the protease production during fermentation was negligible and was constant during whole fermentation cycle. Hence, there could role of other factors in the reduced activity after 3rd day of fermentation.

In the present study, the cell-free culture supernatant of the organism was tested for antibacterial activity and susceptibility to enzymes, organic solvents, and to high and low temperature. The antimicrobial activity of *B. subtilis* KBB was partially susceptible to proteinase K, pronase and chymotrypsin, suggesting that it is proteinaceous, as described previously (Jack *et al.* 1995; Korenblum *et al*. 2005). The common organic solvents such as methanol, ethanol, chloroform etc. did not affect the activity of the antimicrobial compound as reported previously (Korenblum *et al*. 2005).

Present study showed that the antimicrobial compound produced by *Bacillus subtilis* KBB seems to be highly thermostable and found active over a wide range of acidic and basic pH. The activity of culture supernatant was not affected by heating below 60°C but slightly reduced activity was observed after heating the supernatant at 100°C. However, the inhibitory activity of the antimicrobial compound was lost after autoclaving (121°C for 15 min). Similar to the results of the present study, production of thermo-stable antimicrobial substances with stable activity at broad range of pH by *Bacillus* spp. in culture supernatant has been widely reported previously (Phae *et al*. 1990; Leddabi *et al*. 1994; Doss and Tyejegaja 1996; Motta and Brandelli 2002). Similarly, Bacillin, produced by *B. subtilis* retains antimicrobial activity even after heating at 100ÚC for 30 min. under acidic conditions (pH 6.5) (Stein 2005). The peptide antibiotic ericin S remains fully stable and bioactive even after heating at 60ÚC, but gradually lost its activity at temperature above 60ÚC, and lost 80% antimicrobial activity after heating at 100ÚC for 90 min. (Stein *et al.* 2002). Regarding the storage stability and shelf life, the antimicrobial compound does not undergo any kind of degradation during storage at cold temperature for a month and at room temperature for 4 month.

In the present study, extraction of antimirobial compound was optimized by one-step solvent extraction procedure using Bligh and Dyer method (1959) with slight modification. In this method the proportions (Metahanol 4: supernatant 2 : chloroform 1) of both the methanol and chloroform should be accurate otherwise the precipitate residue cannot be obtained. The partial purification of antimicrobial compound was performed with successive solvent washing. Protein nature of the partially purified compound was confirmed by Biuret test and Ninhydrin test. The purity of the compound was confirmed by TLC as only one spot (Rf=0.5) was observed with TLC and bioactivity was confirmed by bioautography. It can be concluded that the method of extraction and purification followed in present study resulted in extraction of single bacteriocin (though it has been considered as partially purified antimicrobial compound). MIC of the compound was 125 ìg/ml for *S. aureus* and 250 ìg/ml for *Salmonella* Typhi W7 indicates broad spectrum but more suitable for gram-positive bacteria.

In present study, although GCMS and NMR, UV, IR spectrum all are suggesting the peptide nature of the compound (The spectra are presented in annex 3) but complete structure elucidation was not done. The molecular weight of antimicrobial compound was aprox.14 KD as revealed by SDS page. However, complete chemical formula of the bioactive molecule could not be elucidated in present study.

REFERENCES

Abee, T. (1995) Pore forming Bacteriocins of Gram-positive Bacteria and Self Protection Mechanisms of Producer organisms. *FEMS Microbiol Lett* 129**,** 1–10.

Barefoot, S.F. and Nettles, E.G. (1993) Antibiosis revisited: bac-teriocins produced by dairy starter cultures. *J Dairy Sci* 76, 2366-2379.

Bauer, A. W., Kirby, M. W., Sherris, J. C. and Turck, M. (1966) Antibiotic susceptibility testing by a standardized single disc method. *Am J Clin Pathol* 45, 493-496.

Berdy, J. (1974). Recent developments of antibiotic research and classification of antibiotics according to chemical structure. *Adv Appl Microbiol* 18, 309–406.

Berkeley, R.C.W., Logan,N.A., Shute, L.A and Capey, A.G. (1984) Identification of *Bacillus* species In: Methods in microbiology vol, 16, pp. 292-323, Acedemic press, London.

Bie, X., Lu, Z. and Lu, F. (2006) Preservative Effect of an Antimicrobial Substance from *Bacillus subtilis* fmbJ on Pasteurised Milk During Storage. *Food Sci Technol International* 12, 189-194.

Bizani, D., Motta, A.S., Morrissy, J.A.C., Terra, R.M.S., Saouto A.A., Brandelli, A. (2005) Antibacterial activity of cerein 8A, a bacteriocin-like peptide produced by *Bacillus cereus*. *International Microbiol* 8, 125-131.

Braoudaki, M., Hilton, A. C. (2004). Adaptive Resistance to Biocides in Salmonella enterica and Escherichia coli O157 and Cross-Resistance to Antimicrobial Agents. *J. Clin. Microbiol.* 42: 73-78.

Buswell, C. M., Herlihy, Y. M., Lawrence, L .M., McGuiggan, J. T M., Marsh, P. D.,Keevil, C. W.,and Leach, S.A. (1998) Extended Survival and Persistence of *Campylobacter spp.* in Water and Aquatic Biofilms and Their Detection by Immunofluorescent-Antibody and -rRNA Staining. *Appl Environ Microbiol.* 64, 733–741.

Carrillo, C., Teruel, J.A., Aranda, F.J., and Ortiz, A. (2003) Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. *Biochim Biophys Acta* 1611: 91–9.

Chopra, I., Hodgson, J., Metcalf, B. and Poste, G. (1997) The search for antimicrobial agents effective against bacteria resistant to multiple antibiotics. *Antimicrob Agents Chemother* 41, 497-503.

Chopra, I.,Hesse, L., and O'Neil, A.J. (2002)Exploiting current understanding of antibiotic action for discovery of new drugs. J Appl Microbiol, 92 (s1), 4S–15S.

Claus, D., and Berkeley, R. C. W. (1986) Genus Bacillus Cohn1872, 174 In : Bergey's Manual of Systematic Bacteriology vol. 2 ed. Sneath, P. H. A. Mair, N. S. Sharpe, M. E. and Holt J. G. pp 1104-1139. The Williams & Wilkins Co., Baltimore.

Davidson, P.M. and Harrison, M.A. (2002) Resistance and adaptation to food antimicrobials, sanitizers, and other process controls. *Food Technol* 56**,** 69-78.

Doss, C. and Tejegya, R. (1996) growth and suppression of some wood decay fungi by *Bacillus subtillis*. *Australian J Bot* 44, 705-112.

Fox, S.L., and Bala, G.A. (2000) Production of surfactant from *Bacillus subtilis* ATCC 21332 using potato substrates. *Bioresour Technol* 75, 235–240.

Gilbert, P., Allison, D. G. and McBain, A. J. (2002)Biofilms in vitro and in vivo, do singular mechanisms imply cross-resistance?. *J Appl Microbiol* 92, 98S-110S.

Gilbert, P., McBain, A. J. (2003). Potential Impact of Increased Use of Biocides in Consumer Products on Prevalence of Antibiotic Resistance. *Clin. Microbiol. Rev.* 16: 189-208.

Hancock, R. E. W. and Chapple, D. S. (1999) Peptide antibiotics. *Antimicrob Agents Chemother* 43**,** 1317-1323.

Hara, T., and Ueda, S. (1982) Regulation of polyglutamate in *Bacillus subtilis* (natto): transformation of high PGA productivity *Agric Biol Chem* 46, 2275–2281.

Inaoka, T., Takahashi, K., Ohnishi-Kameyama, M., Yoshida, M., and Ochi, K. (2003) Guanine nucleotides guanosine 5'-diphosphate 3'-diphosphate and GTP co-operatively regulate the production of an antibiotic bacilysin in *Bacillus subtilis*. *J Biol Chem* 278, 2169–2176.

Inaoka, T., Takahashi, K., Yada, H., Yoshida, M., and Ochi, K. (2004) RNA polymerase mutation activates the production of a dormant antibiotic 3,3'-neotrehalosadiamine via an autoinduction mechanism in *Bacillus subtilis*. *J Biol Chem 279*, 3885–3892.

Jack, R. W., Tagg, J. R. and Ray, B. (1995) Bacteriocins of gram-positive bacteria. *Microbiol Rev* 59, 171-200.

Jansen, E. F., and Hirschmann, D. J. (1944) Subtilin, an antibacterial factor of *Bacillus subtilis*: culturing condition and properties. *Arch. Biochem* 4, 297-309.

Katz, E. and Demain, A. L. (1977). The peptide antibiotics of Bacillus: chemistry, biogenesis and possible functions. *Bacteriol Rev* 40, 449–474.

Klaenhammer, T. R. (1993) Genetics of bacteriocins by lactic acid bacteria. *FEMS Microbiol Rev* 12, 39-86.

Korenblum,E., von der Weid, I., Santos, A.L.S., Rasado, A.S., Sebastian, G.V., Coutinho, C.M.L.M., Magalhaes, F.C.M., de Paiva, M.M and Seldin, L. (2005) Production of antimicrobial substances by *Bacillus subtilis* LFE1, *B.firmus* H2O-1 and *B. licheniformis* T6-5, isolated from an oil reservoir in Brazil. *J Appl Microbiol* 98, 667-675.

Leclere,V., Marti,R., Bechet.M., Fickers.P. and Jacques, P. (2006) The lipopeptides mycosubtilin and surfactin enhance spreading of Bacillus subtilis strains by their surface-active properties. *Arch Microbiol* 186, 475-481.

Leddabi, M., Galvez, A., Maqueda, M., Martinez-Bueno, M. and Valdivia, E. (1994) Fungicin M4: a narrow spectrum peptide antibiotic from Bacillus licheniformis M-4. *J Appl Bacteriol* 77, 49–53.

Maillard, J.-Y. (2002) Antibiotic and biocide resistance in bacteria: perceptions and realities for the prevention and treatment of infection. *J Appl Microbiol* 92, viiS.

Motta, A.S. and Brandelli, A. (2002) Characterization of an antibacterial peptide produced by *Brevibacterium linens*. *J Appl Microbiol* 92, 63-70.

Mulligan, C.N., Yong, R.N., and Gibbs, B.F. (2001) Surfactant enhanced remediation of contaminated soil: a review. *Eng Geol* (Amsterdam) 60, 371–380.

NCCLS (1997) National Committee for Clinical Laboratory Standards (M 2-A6) Performance Standards for Antimicrobial Disc Susceptibility Tests, 6th edn. PA, USA: Wayne.

NCCLS (1997), National committee for Clinical Laboratory Standards (M 7-A4), Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobicalyly.4th ed.

NCCLS (2002) Performance Standards for Antimicrobial SusceptibilityTesting; Twelfth Informational Supplement 2002. NCCLS Document M100-S12. PA, USA: Wayne.

Newton, B. A. (1956) The properties and mode of action of the polymyxins. *Bact Rev* 20, 14-18.

Nicholson, W.L. (2002) Roles of *Bacillus* endospores in the environment. *Cell Mol Life Sci* 59, 410–416.

Paik, H. D., Lee, N.K., Lee, H.K., Hwang, Y.I. and Pan, J. C. (2000) Identification and partial characterization of cerein BS229, bacteriocin produced by *Bacillus cereus* BS229. *J Microbiol Biotechnol* 10**,** 195-200.

Pattnaik, P., Kaushik, J. K. Grover, S. and Batish, V. K. (2001) Purification and characterization of a bacteriocin-like compound (lichenin) produced anaerobically by *Bacillus licheniformis* isolated from water buffalo. *J Appl Microbiol* 91**,** 636-645.

Peypoux, F., Bonmatin, J.M., and Wallach, J. (1999) Recent trends in the biochemistry of surfactin. *Appl Microbiol Biotechnol* 51, 553–563.

Pinchuk, I.V., Bressollier, P., Sorokulova, I.B., Verneuil, B., and Urdaci, M.C. (2002) Amicoumacin antibiotic production and genetic diversity of *Bacillus subtilis* strains isolated from different habitats. *Res Microbiol* 153, 269–276.

Priest, F. G. (1993) Systematics and ecology of *Bacillus*, pp 3-16, *In* Sonenshein, A. L., Hoch, J. A., and Losick, R. (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.

Priest, F. G. and Girgorova, R. (1991) Methods for studying the ecology of endospore-forming bacteria, pp 565-591,*In* Grigorova, R. and Norris, J. R. (ed.), Methods in microbiology, vol 22, Academic Press, London, U.K.

Rosenberg, E., and Ron, E.Z. (1999) High and low-molecular-mass microbial surfactants. *Appl Microbiol Biotechnol* 52, 154–162.

Sahl, H.G, and Bierbaum, G. (1998) Lantibiotics: Biosynthesis and Biological Activities of Uniquely Modified Peptides from Gram-Positive Bacteria Annu. Rev Microbiol 52, 41–79.

Sahl, H.G., Jack, R.W., Bierbaum, G. (1995) Biosynthesis and biological activities of lantibiotics with unique post-translational modifications. *Eur J Biochem* 230, 827–53.

Schallmey, M., Singh, A. and Ward, O.P. (2004) Developments in the use of *Bacillus* species for industrial production. *Can J Microbiol* 50, 1–17.

Seah, A. H. L., Teo, A. Y.L. and Tan, H.M. (2002) Antimicrobial compounds from *Bacillus subtilis* for use against animal and human pathogens. U.S. Patent and Trademark Office serial no. 10/306,365.

Stackbrandt, E.,and Goebel,B.M.(1994) taxonomic note A place for DNA DNA reassociation and 16SrNA sequence analysis in the present species definition in bacteriology .*Int Jsyst Evol Microbiol* 44, 846-849.

Stein, T. (2005) *Bacillus subtilis* antibiotics: structures, syntheses, and specific functions. *Mol Microbiol* 56, 845–857.

Stein, T., Borchert, S., Conrad, B., Feesche, J., Hofemeister, B., Hofemeister, J., and Entian, K. D. (2002) Two different lantibiotic-like peptides originate from the ericin gene cluster of *Bacillus subtilis* A1/3. *J Bacteriol* 184, 1703–1711.

Steinborn, G., Hajirezaei, M.R. and Hofemeister, J. (2005) *Bac* genes for recombinant bacilysin and anticapsin production in *Bacillus* host strains. *Arch Microbiol* 183, 71–79.

Stickler D.J.(2002)Susceptibility of antibiotic-resistant Gram-negative bacteria to biocides: a perspective from the study of catheter biofilms. *J Appl Microbiol* 92, 163S–170S.

Stover, A.G. and Driks, A. (1999) Regulation of synthesis of the *Bacillus subtilis* transition-phase, spore-associated antibacterial protein TasA. *J Bacteriol* 181, 5476–5481.

Tamehiro, N., Okamoto-Hosoya, Y., Okamoto, S., Ubukata, M., Hamada, M., Naganawa, H., and Ochi, K. (2002) Bacilysocin, a novel phospholipid antibiotic produced by *Bacillus subtilis* 168. *Antimicrob Agents Chemother* 46: 315–320.

Teo, Y.A. and Tan, H.M (2005) Inhibition of *Clostridium perfringens* by a Novel Strain of *Bacillus subtilis* Isolated from the Gastrointestinal Tracts of Healthy Chickens. *Appl Environ Microbiol* 71, 4185-4190.

Wei, Y.H., Chu, I.M. (2002) Mn^2 + improves surfactin production by *Bacillus subtilis*. *Biotech Lett* 24**,** 479–482.

Yazgan, A., Cetin, S., and Ozcengiz, G. (2003) The effects of insertional mutations in *comQ, comP, srfA, spo0H, spo0A* and *abrB* genes on bacilysin biosynthesis in *Bacillus subtilis*. *Biochim Biophys Acta* 1626, 51–56.

Yoshida,S., Hiradate, S., Tsukamoto, T., Hatakeda, K. and Shirata, A. (2001) Antimicrobial Activity of Culture Filtrate of *Bacillus amyloliquefaciens* RC-2 Isolated from Mulberry Leaves. *American Phyto path Society* 91, 181- 187.

Zheng, G. and Slavic, M.F. (1999) Isolation, partial purification and characterization of a bacteriocin produced by a newly isolated Bacillus subtilis strain. *Lett Appl Microbiol* 28, 363–367.

***