Antagonistic behaviour of organic compounds from *Bacillus* species and *Brevundimonas* specie

Hina Sadiq and Nazia Jamil*
Department of Microbiology and Molecular Genetics, University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan

Abstract: Bacterial strains, *Bacillus cereus* Lb (KF011486), *Brevundimonas* sp. A2 (JX996070), *Bacillus cereus* AZS and *Bacillus* sp. 11A, isolated from soil sample, were checked for their antimicrobial property against *Bacillus* as test organism. The bactericidal effect of the antagonistic strains against test organism was found to be at 1280, 1280, 40 and 160 arbitrary units (AU/ml), respectively. The Crude Antimicrobial Compound (CAC) had a bactericidal effect on target cell by degeneration of its cell wall. The chemical analysis of TLC purified extract of intracellular and extracellular antimicrobial compound produced by *Bacillus cereus* Lb by GC-MS analysis revealed the presence of organic compounds such as acetic acid and certain volatile organic substances such as, toluene, 2-butanone, etc., with antimicrobial property. N-acetylmuramoyl-L-alanine amidase is a cell wall hydrolysing enzyme and involved in cell wall degeneration of the target cells. These volatile organic compounds help this enzyme by decreasing the pH of the environment hence maximizing the amidase activity which possesses maximum activity at pH range of 5.5-6.5.

Keywords: Antagonistic behaviour, N-acetylmuramoyl-L-alanine amidase, toluene, 2-butanone.

INTRODUCTION

Current studies have discovered that volatile organic compounds (VOCs) produced by some antagonistic bacteria have antibacterial and antifungal properties. In 2012, Effmert described 300 bacteria and fungi as VOC producers. About 671 VOCs produced by 212 bacterial species. Bacterial VOCs play a beneficial role in three ways: promoting plant growth, inhibiting the growth of plant pathogens and inducing systemic resistance. Due to several unwanted health effects produced by artificial additives, many consumers are interested in the replacement of potentially harmful artificial ingredients with safe and natural compounds. As such, there is growing concern in expanding the use of effective natural antimicrobials such as thymol, carvacrol and bacteriocins as preservative agents (Olasupo *et al.,* 2003) Many bacteria produce variety of wide spectrum antimicrobial compounds such as organic acids and lytic agents such as lysozyme. Moreover, some bacteria produce peptide antimicrobial compounds such as bacteriocins with narrow spectrum of activity (Motta *et al.,* 2004). *Bacillus cereus*, one of around 60 representatives of the widely varied *Bacillus* genus, comprises the so called "*Bacillus cereus* group" along with the very similar species *B. mycoides*, *B. thuringiensis* and *B. anthracis*. These four species only slightly differ from each other (Torkar & Matijasic, 2003). *Bacillus* is a remarkable genus to explore antimicrobial activity since *Bacillus* species produce a large number of peptide antibiotics representing several different basic chemical structures (Hamdache *et al.,* 2013). The ability of *Bacillus* species to produce antibiotics has been acknowledged for more than 50 years and peptide's antibiotics represent the leading class (Ghanbari *et al.,* 2009). Production of bacteriocins or bacteriocin-like substances had been reported in many species of the genus *Bacillus* including *B. subtilis, B. coagulans, B. cereus, B. thuringiensis, B. megaterium* and others making them attractive candidates for biological control agents. Potential bacteriocins from *Bacillus species* have low allergenic potential, activity at low concentrations and they are easily degraded in the gastrointestinal tract (Leite *et al*., 2016). It is well known that most bacterial species are capable of producing a diverse array of molecules in the course of their growth *in vitro* (and most probably also in their natural habitats) that may be inhibitory to other bacteria (Torkar & Matijasic, 2003). The purpose of this study was to screen and characterize bacteriocin producing bacterial strains associated with indigenous soil environment. Measuring growth phase-related bacteriocin synthesis of the bacterial strains. Confirming the bacteriocin-like character of their crude extract by preliminary detecting their antagonistic activity against indicator strain. Checking stability of crude extract of bacteriocin. The antimicrobial properties of *Bacillus* spp. and *Brevundimonas* sp. with their mode of action under microscope. Partially purified compounds from TLC was analysed by GC/MS.

MATERIALS AND METHODS

Sample collection and purification of bacterial strains

Samples from various sources like dry surface soil and soil from rhizosphere were collected from Punjab University. Strains were purified by streak plate method. *Bacillus cereus* Lb, *Brevundimonas* sp. A2, *Bacillus cereus* AZS and *Bacillus* sp. 11A were isolated from soil samples from university area and used as antagonistic

**Corresponding author:* e-mail: jamil_nazi@yahoo.com

strains (Aslam and Jamil, 2015). *Bacillus* sp. was used as indicator strain. The antimicrobial strains were grown in Luria-Bertani (LB) agar at 37°C under aerobic condition and kept at 4°C.

Detection of antimicrobial activity

The antimicrobial activity was detected by agar well diffusion method (Tagg & McGiven, 1971) for *Bacillus cereus* Lb, *Brevundimonas* sp. A2, *Bacillus cereus* AZS and *Bacillus* sp. 11A. The antagonistic zones (in mm) were measured after 24h incubation. *Bacillus* sp. was used as indicator strain. The antimicrobial compound producing strains were grown in Luria-Bertani (LB) agar at 37°C under aerobic condition and kept at 4°C. As a blank same media without inoculation was used.

Quantification of antimicrobial activity

The antimicrobial activity of the strains was tested by two-fold serial dilutions (Bhatta & Kapadnis, 2010). The antagonistic activity was expressed in terms of Arbitrary Units (AU). One arbitrary unit was defined as the reciprocal of the highest dilution of the supernatant that inhibited target organism x 1000, divided by the volume of supernatant added to the well.

Time profiling and temperature stability of antimicrobial compound

The time course of antimicrobial compound production by the producer strains was studied in two different media i.e., LB broth and Brain Heart Infusion (BHI) broth, for 40h against target organism *Bacillus*. The producer strains were grown in 50ml LB and BHI broth with constant agitation at 150 rpm. The average antagonistic activity against indicator strain and O.D.600nm of both media was determined at 2h intervals using UV-visible spectrophotometer (IRMECO, U2020). Moreover, effect of temperature on the stability of bacteriocin was also determined at three different temperatures (0˚C, 20˚C and 37˚C).

Partial purification of antimicrobial compound

The antagonistic strains were grown at 37°C overnight with constant agitation. Cells were removed by centrifugation at 14800rpm for 10 minutes. The supernatant was separated for extraction of extra cellular antimicrobial compound while pellet was kept for the extraction of intracellular antimicrobial compound.

The supernatant and resuspended pellet was extracted with equal volume of ethyl acetate. The organic phase was separated and evaporated under reduced pressure. The resulting powder was named as Crude Antimicrobial Compound (CAC), dissolved in 1X Phosphate Buffer Saline (PBS) and stored in a clean glass vial for further processing.

Mode of action of antimicrobial compound

Bacteriocin in CAC was determined by dissolving it in 1X Phosphate Buffer Saline (PBS). The indicator cells were harvested from early stationary-phase. Cells were incubated with antimicrobial compound for 1h. After incubation, the cells were stained with 1% crystal violet for 1 minute and examined under metallurgy microscope (Meiji-Techno MA927/05, Japan) for morphological differences.

Thin layer chromatography

An aliquot (5µl) of intracellular and extra cellular crude antimicrobial compound was spotted on TLC plates (Merck TLC Aluminium sheet 20×20cm Silica Gel 60 F_{254}) and separated with ethylacetate/hexane (1:1) solvent system. After drying, plates were stained with iodine and observed for bands. Developed and air dried plates were also visualized using UV light at 254 nm and 366 nm. Components showing UV absorbance and fluorescence were marked and scanned. Moreover, the separated bands were checked for the antimicrobial property and their Retention value (R_f) was calculated.

Gas chromatography mass spectrometry

The *Bacillus cereus* Lb intracellular and extracellular bands of TLC with antimicrobial activity were analysed using GC/MS. A gas chromatograph (QP2010, Shimadzu, Japan) was interfaced to an Electron Ionization (EI) mode and analysis was done by nonpolar HP-5 (Hewlett Packard). The capillary column was a 30m with film thickness of 0.25µm. The gas chromatograph oven temperature was held at 80°C for 1min and then increased to 300°C at 5°C min^{-1}. Helium was used as carrier gas. The injection volume was 1µl. In the identification, the mass spectra library, National Institute of Standards and Technology (NIST27 and NIST147) was used. The identification was based on 90% similarity between the spectra of unknown and reference.

RESULTS

Isolation and screening

A total of 10 bacterial strains were isolated from various sources and screened for antibacterial activity against target organism. Out of 10 isolates, 4 showed antibacterial activity against target organism. These four isolates were selected and then used for further studies.

Detection and quantification of antimicrobial activity

All the strains showed significant antimicrobial activity against the test organism *Bacillus*. The antimicrobial activity of the strains was determined in terms of arbitrary units (AU/ml) as shown in table 1. *Bacillus cereus* Lb showed very significant activity against *Bacillus* which was approximately 1289 AU/ml. The activity of other strains *Brevundimonas* sp (JX996070) A2, *Bacillus cereus* AZS, *Bacillus* sp. 11A was 1280, 40, 160 AU/ml.

Time profiling and temperature stability of antimicrobial compound

The time profiling and temperature stability of the antimicrobial compound was determined using two

Table 1: Nature of compounds from the bioactive fraction of the extracellular antimicrobial compound from *Bacillus cereus* Lb as compared to NIST147 and NIST27 library

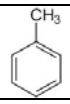
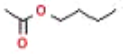
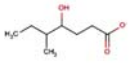
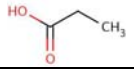
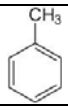
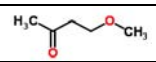
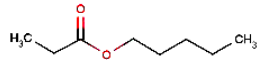
Sr. No.	Compound	Molecular Formula	Molecular Weight	Structural Formula
1-	Toluene	C_7H_8	92	
2-	Acetic Acid, butyl ester	$C_6H_{12}O_2$	116	
3-	2-Pentanol, acetate	$C_7H_{14}O_2$	130	
4-	Propanoic Acid	$C_8H_{14}O_3$	158	

Table 2: Nature of compounds from the bioactive fraction of the intracellular antimicrobial compound from *Bacillus cereus* Lb as compared to NIST147 and NIST27 library

Sr. No.	Compound	Molecular Formula	Molecular Weight	Structural Formula
1-	Toluene	C_7H_8	92	
2-	1,3-propanediol	$C_5H_{12}O_2$	104	
3-	4-methoxy-2-butanone	$C_5H_{10}O_2$	102	
4-	Acetic acid, pentyl ester	$C_7H_{14}O_2$	130	

growth media for 40 h. More growth was observed in Brain Heart Infusion broth as compared to Luria bertani broth and the antagonistic activity was observed up to 40 h (fig. 1). Moreover, the cell free supernatant retained its activity at 20°C and 37°C (fig. 2).

Partial purification of antimicrobial compound

Ethyl acetate was used for partial purification of intracellular and extracellular antimicrobial compounds. These crude antimicrobial compound (CAC) was tested against *Bacillus* sp. These CACs gave positive antimicrobial activity and they were also temperature stable at room temperature.

Mode of action under metallurgy microscope

The cells of the test organism *Bacillus* from the early stationary phase culture were harvested. After incubation with the crude antimicrobial compound for 1 h, cells were observed under microscope. The test organism cells showed vesiculization of protoplasm and pore formation as shown in fig. 3.

Thin layer chromatography

Different bands observed after UV analysis were checked for antimicrobial property (fig. 4). Only one band in each sample possessed antimicrobial property The retention values (R_f) of antimicrobial active fractions produced by *Bacillus cereus* Lb, *Brevundimonas* sp. A2, *Bacillus cereus* AZS, and *Bacillus* sp. 11A for Intracellular were; 0.42, 0.7, 0.42, respectively and for extracellular; 0.52, 0.8, 0.5, and 0.64 respectively.

GC/MS

The peaks obtained after GC/MS of extracellular and intracellular purified antimicrobial fraction of *Bacillus cereus* Lb were analysed (fig. 5) and compared with NIST27 and NIST147 library which showed that the compounds were volatile organic acids (tables 1 and 2). The organisms showed diversity between extra as well as intracellular compounds. The extracellular compounds included toluene, Acetic Acid-butyl ester, 2-Pentanol-acetate and Propanoic Acid with retention time 2.083, 2.267,2.492 and 2.742 respectively, while the intracellular compounds included Toluene, 1,3-propanediol, 4-methoxy-2-butanone, and Acetic acid-pentyl ester with retention time 2.075, 2.258, 2.483, and 2.733 respectively (Tait *et al.*, 2014).

DISCUSSION

Most of the bacteria commonly produce antimicrobial compounds. An impressive collection of microbial defence system is formed by bacteria. It included wide-spectrum traditional antibiotics, metabolic by-products such as VOCs, and lytic enzymes such as lysozyme. In fact some certain biologically active peptide moieties with bactericidal method of action were also included. Example of several types of proteins were exotoxins

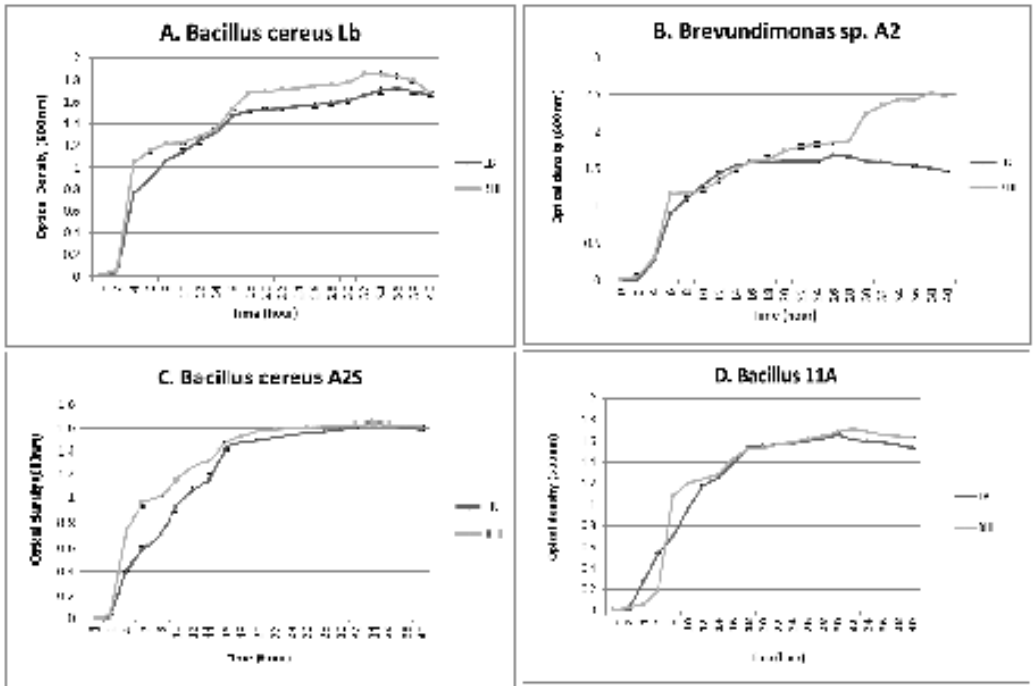


Fig. 1: Growth pattern of *Bacillus cereus* Lb (A), *Brevundimonas* sp. A2 (B), *Bacillus* AZS (C) and *Bacillus* 11A (D) in LB and BHI media. Increased growth rate was observed in BHI broth as compared to LB broth in all strains.

(Riley and Wertz, 2002; Yeaman and Yount, 2003). There are unique microorganisms, especially *Bacillus* yields products found in microbiologically unexplored ecosystems around the world suggest that a careful exploration of other habitats might continue to be useful (Sihem *et al.*, 2011).

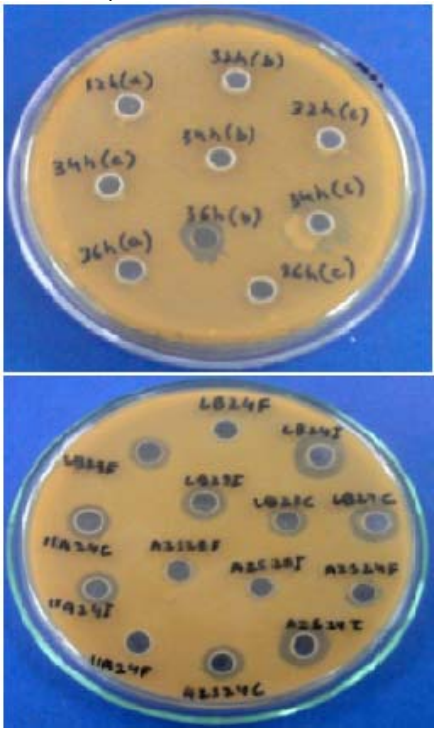


Fig. 2: Temperature stability of the bacteriocin produced by antagonistic strains against test organism. (A) Stability of A2 bacteriocin after incubation at 37°C as indicated by arrow. No zone was observed in case of 0°C and 20°C. (B) Increased stability of Lb and AZS bacteriocin after incubation at 37°C (I) as compared to 20°C (C) indicated by solid arrow. No stability was observed at 0°C (F).

The production of volatile acids by *Bacillus* sp to reduce the viability of plant pathogens such as *Xanthomonas* have been reported by Xie *et al.* (2018). Current study was based on the characterization of antimicrobial behaviour and properties of antimicrobial compounds produced by members of genera *Bacillus* and *Brevundimonas* JX996070 (A2). This study has provided an insight into the bacteriocin production of *Bacillus* sp and their role in bio control. These can retain their activity under various temperatures and pH (Aslam and Jamil 2015).

The cells of the test organism *Bacillus* from the early stationary phase culture were harvested. After incubation with the crude antimicrobial compound for 1 h, cells were observed under microscope. The test organism cells showed vesiculization of protoplasm and pore formation as shown in fig. 3. Our results suggest that the antimicrobial compounds produced by the antagonistic strains had bactericidal mode of action against *Bacillus* as test organism based on the microscopy of treated cells which showed pore formation in target cell wall. Cell wall hydrolysis is an important mechanism which antagonistic strains possess against target organisms (Snyder and Worobo, 2013). It was also suggested that change in pH might observed due to the production of volatile organic

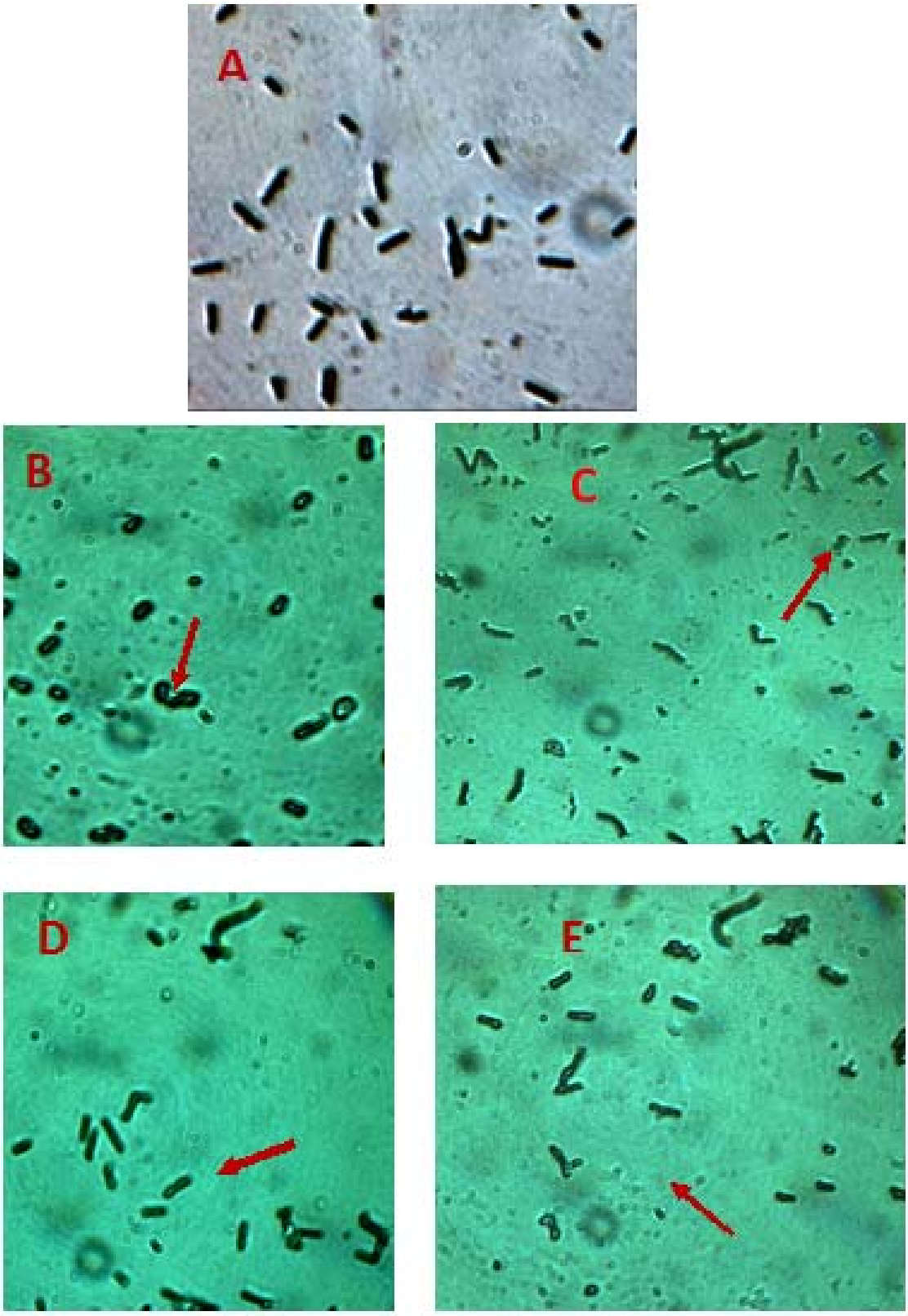


Fig. 3a: Microscopy of cells of test organism (*Bacillus*) with and without treatment with intracellular and extra cellular bacteriocin. Cells of test organism after treatment with extra cellular and intracellular Lb bacteriocin (B & C), extra cellular and intracellular A2 bacteriocin (D & E) showed cell wall degeneration and altered morphology as compared to control untreated test organism cells (A).

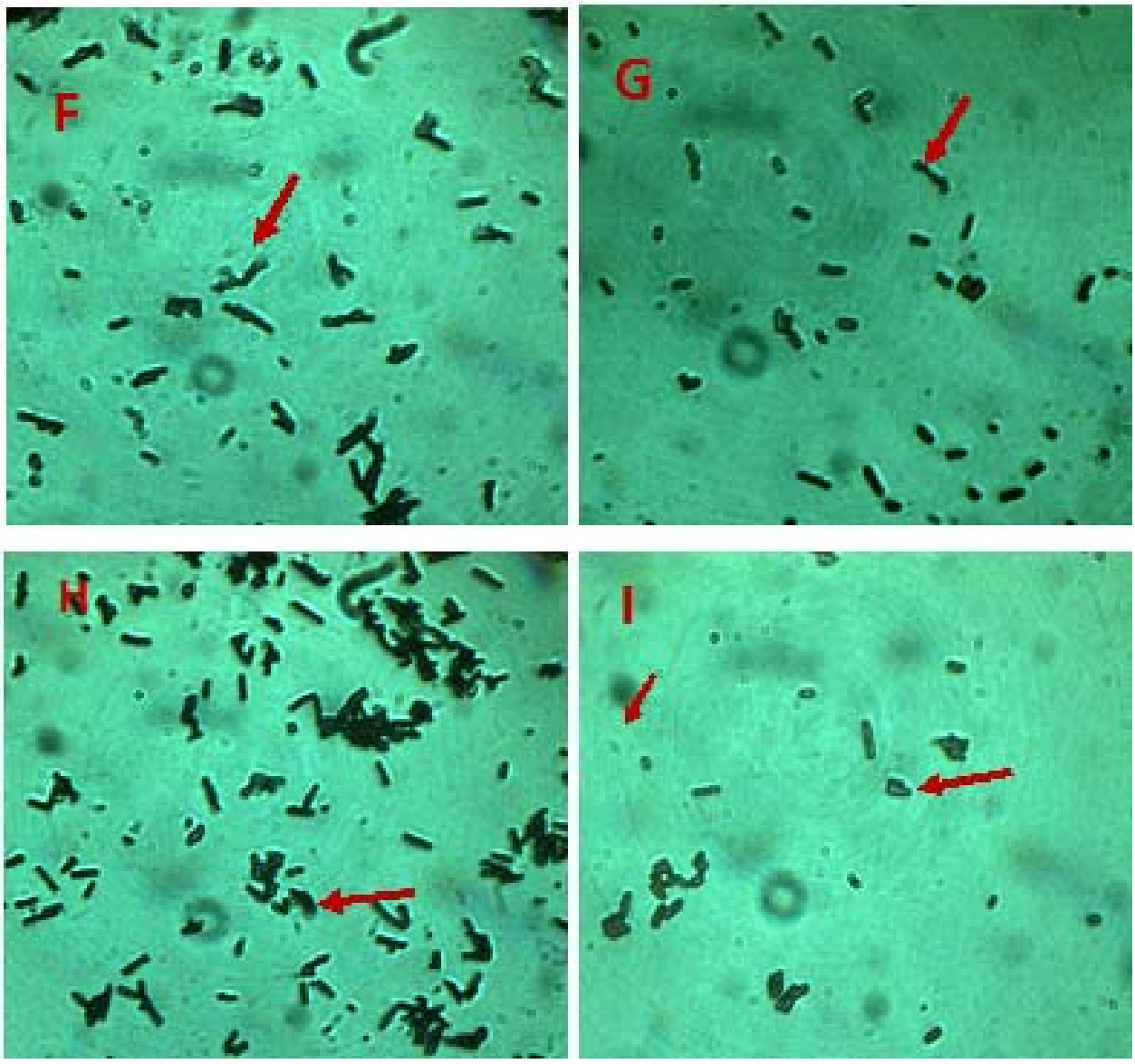


Fig. 3b: Microscopy of cells of test organism (*Bacillus*) with and without treatment with intracellular and extra cellular bacteriocin (continued). Cells of test organism after treatment with extra cellular and intracellular AZS bacteriocin (F & G) and extra cellular and intracellular 11A bacteriocin (H & I) showed showed cell wall degeneration and altered morphology as compared to control untreated test organism cells (A).

compounds (Raza, Wei *et al*. 2016). These VOCs were characterized by GC/MS analysis in which acetic acid production observed in intra and extracellular bio-active fractions. Being a weak acid it slightly reduces pH and this pH change targets hydrolytic activity of N-acetylmuramoyl L-alanine amidase. So, these VOCs, on one hand, are liable for antimicrobial activity by diffusing inside the target bacterial cells. This resulting in disruption of the ionic balance inside the cell. While on the other hand it creating a decrease pH environment for N-acetylmuramoyl L-alanine amidase to perform its cell wall hydrolytic activity. So, a collective and an enhanced antimicrobial effect were observed in the presence of VOCs and N-acetylmuramoyl L-alanine amidase (Raza, Wei *et al*. 2016). *Bacillus* species produced antimicrobial compounds, under study prove to be effective against Gram positive organisms. Genetic determinants of bioactive antimicrobial compound also provided insight into the structure and function relationships.. Present study will cover the way for exploring the antagonistic behaviour of *Bacillus* and their large scale production against Gram positive organisms specifically *Bacillus* species.

Moreover the use of these volatile organic compounds has also been suggested, the bacteria can be used to create an acidic environment which can prove to be a natural food preservative (Crowley *et al.*, 2013). These microbial

volatiles can be exploited for their beneficial and environmentally friendly roles, such as induced systemic resistance against plant pathogens and abiotic factors, plant growth enhancement and antagonistic action against plant pathogenic fungi and nematodes.

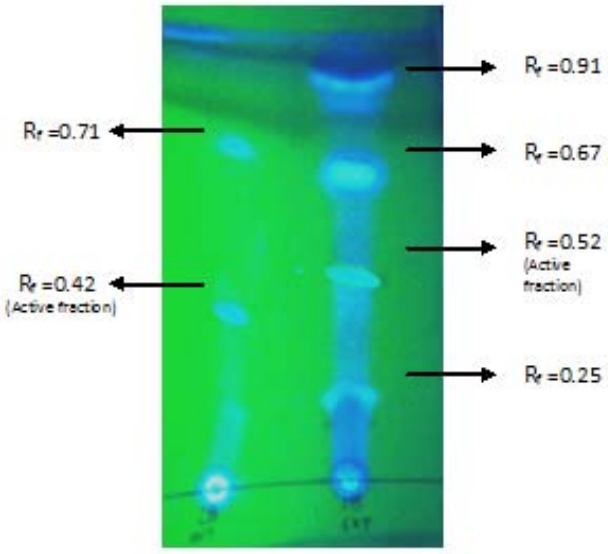


Fig. 4: TLC of crude antimicrobial compound of intracellular (lane A) and extra cellular (lane B) bacteriocin from *Bacillus cereus* Lb *Merck TLC Aluminium sheet 20×20cm Silica Gel 60 F_{254} was used. R_f stands for "Retardation factor"

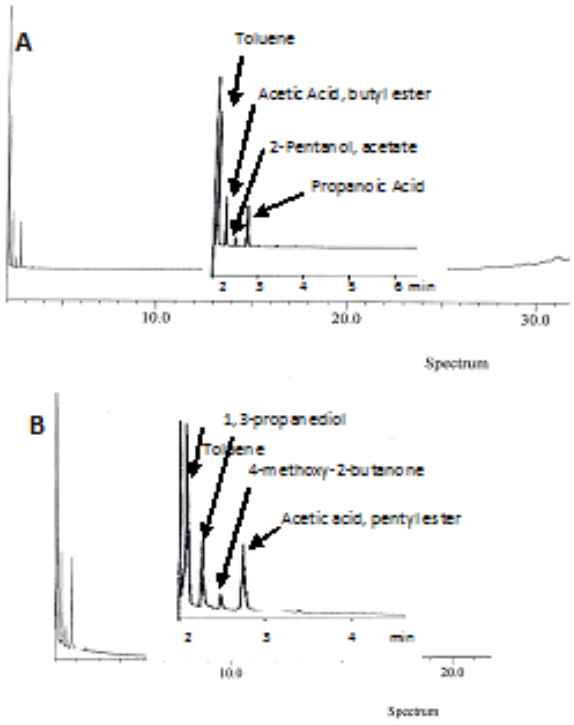


Fig. 5: GC-MS spectra of *Bacillus cereus* Lb intracellular and extra cellular purified antimicrobial compound. GC profile of *Bacillus cereus* Lb intracellular (A) and extra cellular (B) antimicrobial compound purified by TLC and analysed by GC/MS QP2010 from Schimadzu. Inset: detailed overview of individual peak.

CONCLUSION

The antimicrobial compounds, produced by *Bacillus* species, under study prove to be effective against Gram positive organisms. Intracellular and extra cellular volatile organic compounds (VOCs) produced by various bacteria have significant potential to enhance plant growth and control phytopathogens. This work will pave the way for exploring the antagonistic behaviour of VOCs and large scale production of these.

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