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Antibacterial potential of a small peptide from *Bacillus* sp. RPT-0001 and its capping for green synthesis of silver nanoparticles

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Infirmity and death from diseases caused by unsafe food are a continual hazard to communal health safety and socio-economic growth throughout the world. Chemical preservatives are associated with health hazards and toxicity issues. In the study reported here, 200 soil isolates from Western Himalayan region in India were screened for potential antibacterial activity against food-borne pathogens. This study led to the isolation of a bacterial strain belonging to the Genus *Bacillus* and was designated as RPT-0001. The associated antibacterial activity was sensitive to pronase E treatment. Bioassay-guided fractionation using reverse phase high performance liquid chromatography (RP-HPLC) led to isolation of the antibacterial peptide designated as RPT-0001. The molecular weight of RPT-0001 was determined by electro-spray ionization mass spectroscopy (ESI-MS) as 276.9 Da. RPT-0001 was inhibitory to both Gram-negative and Gram-positive food-borne bacteria tested. The characteristics of RPT-0001 do not match with that of any other known antibacterial peptides produced by *Bacillus* sp. or related genera. Purified RPT-0001 was successfully used in synthesis of silver nanoparticles effective against food-borne pathogenic bacteria. The antibacterial peptide and silver nanoparticles synthesized utilizing it as a capping and reducing agent hold promising potential in food preservation, in packaging material and as a therapeutic agent in the treatment of food-borne infections.

Keywords: food-borne pathogens, soil bacteria, antibacterial peptide, RP-HPLC, ESI-MS, silver nanoparticles, antibacterial

Introduction

Bacteria are a common cause of food-borne illness due to which diseases acquired by consumption of food contaminated with pathogenic bacteria and/or toxins produced by them are a fundamental concern to public health (Kim *et al.*, 1995). The use of antimicrobial agents for nutritive and therapeutic treatment in food producing animals has caused the emergence of drug resistant food-borne bacteria (Perreten *et al.*, 1997; Glynn *et al.*, 1998; Teuber, 1999; Threlfall *et al.*, 2000; Angulo *et al.*, 2004). Amongst the Gram-negative bacteria causing food borne infections in humans, *Escherichia coli* is a highly successful coloniser of the intestine. *E. coli* O157:H7 has become widely recognized as a very important cause of food-borne illness over the last two decades (Newell *et al.*, 2010). Another significant Gram-negative food-borne pathogen *Chronobacter sakazakii* (previously known as *Enterobacter sakazakii*) causes meningitis, necrotizing enterocolitis, and bacteremia (Gurtler *et al.*, 2005). Its contamination in milk-based powdered infant formulae has been the source of infections that cause sepsis, brain abscess and meningitis in neonates and infants (Gurtler *et al.*, 2005), with mortality up to 80% (Lai, 2001). *Salmonella* spp., cause water-borne gastroenteritis and typhoid fever in humans affecting more than 20 million people worldwide with mortality of 220000 per year (Woc-Colburn and Bobak, 2009; Mathur *et al.*, 2012). Shigellosis, a foodborne outbreak of *Shigella* spp. can also cause serious damage and mortality. *S. dysenteriae* and *S. flexneri* are the predominant species of this genus. (Nandy *et al.*, 2011; Nygren *et al.*, 2013). Among the Gram-positive bacteria causing food-borne infections, spore formers are more troublesome for food industry as they can resist high temperature (Andersson *et al.*, 1995). *Bacillus cereus* and *Clostridium perfringens* are notable spore forming food-borne bacterial pathogens. *B. cereus* causes diarrhoea and vomiting and its pathogenicity is associated with the production of toxins (Bottone, 2010). *C. perfringens* is responsible for a number of clinical conditions mediated through enterotoxin produced *in vivo* during sporulation. Besides spore formers, *Listeria monocytogenes* is an opportunistic gram positive intracellular pathogen responsible for causing listeriosis, a disease which can be fatal for immunocompromised individuals, infants, the elderly and pregnant women (Renier *et al.*, 2011). *Staphylococcus aureus* is another significant gram positive bacteria associated with foodborne infections and outbreak of *S. aureus* can have fatal consequences (Hyeon *et al.*, 2013). Owing to continual hazard to communal health safety caused by the bacterial food-borne pathogens, there is an ever present need to control them to prevent food-borne illness and outbreaks and extend the shelf life of food. Moreover, in the emerging issue of increased multidrug resistant (MDR) properties in food-borne bacterial pathogens (Threlfall *et al.*, 2000), a renewed effort

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should be made to seek effective antimicrobial agents from different sources and with different mode of action (Silver and Bostian, 1993; Sakoulas *et al.*, 2012; Patil *et al.*, 2013)

Various antimicrobial agents including those from bacterial sources have been used to inhibit food-borne bacteria. Bacteria belonging to genus *Bacillus* are known for their ability to produce several antimicrobial compounds (Sharp *et al.*, 1989). Because of their broad spectrum and efficient antibacterial activity, antimicrobial peptides from *Bacillus* spp. have immense prospective for applications in the food, agricultural, and pharmaceutical industries to prevent or control spoilage and pathogenic microorganisms (Lee, 2011). Though soil is generally accepted as their natural reservoir (Baruzzi *et al.*, 2011), they are efficiently capable of colonizing a wide range of environments like air, aquatic environments, vegetable matter, food, meat, and gastrointestinal tracts of various insects and animals (Nicholson, 2002; Baruzzi *et al.*, 2011). Except *Bacillus anthracis* and *B. cereus*, species belonging to *Bacillus* Genus are saprophytic and are not regarded as pathogenic to humans. Like the lactic acid bacteria, the Genus *Bacillus* include representatives like *Bacillus subtilis* that are "generally recognized as safe" (GRAS) (Sharp *et al.*, 1989) and can hence find application in the control of food pathogens and spoilage microorganisms during food processing.

Silver in nano-form has emerged as a promising antibacterial agent and holds promise to target food-born bacterial species (Mohammed Fayaz *et al.*, 2009; Chaloupka *et al.*, 2010; Duncan, 2011). Silver Nano Particles (SNPs) possess antimicrobial efficacy due to the large surface area to volume ratio. Conventionally, chemical reduction by using toxic chemicals such as sodium borohydride ($NaBH_4$), citrate, or ascorbate is the most frequently applied approach for preparation of SNPs. Considering that such reducing agents may be associated with environmental toxicity and biological hazards, the development of a green synthesis approach for SNPs is desired (Sharma *et al.*, 2009).

The study reported here was undertaken to isolate and characterize biomolecules from soil bacteria active against food-borne bacterial pathogens and their application in green synthesis of SNPs that may have potential applications in food preservation, extension of shelf life of food by using as packaging material and as a therapeutic agent in the treatment of food-borne infections. An antibacterial peptide RPT-0001 was characterized based on its spectrum of inhibition and Electrospray Ionization (ESI) Mass Spectrometry (ESI-MS). An eco-friendly and facile approach for synthesis of SNPs using the RPT-0001 purified fraction as a capping agent was also carried out. The antibacterial potential of synthesized SNPs was tested for several Gram-negative and Gram-positive food-borne bacterial pathogens.

Materials and Methods

Bacterial strains, media, and growth conditions

All soil isolates were grown aerobically at 30°C for 24–48 h on tryptone soya (Himedia) medium. All indicator bacteria namely *Listeria monocytogenes* MTCC 657, *Clostridium perfringens* MTCC 450, *Bacillus cereus* ATCC 11778, *Chronobacter sakazaki* MTCC 659, *Salmonella enterica* subsp. *enterica* ATCC 10708, *Bacillus subtilis* MTCC 441, *Micrococcus luteus* ATCC 10240, *Shigella flexneri* ATCC9199, *Staphylococcus aureus* ATCC29213, and *Escherichia coli* strains (ATCC 25922, MTCC946, and MTCC483) were cultivated in Mueller Hinton broth (Merck) while *Candida albicans* ATCC 10231 was cultured in yeast extract peptone dextrose (YEPD) broth (Himedia). Here, ATCC stands for American type culture collection, USA while MTCC stands for Microbial type culture collection, India. Where solid media were required, 1.5% agar (w/v) (Oxoid) was added. The indicator microorganisms were incubated at 37°C with shaking at 200 rpm except for *C. perfringens* which was grown in static conditions.

Enumeration of culturable strains from soil samples and screening for antibacterial activity

One gram of the soil sample was mixed with 9 ml saline [0.85% NaCl (Himedia)] and subsequently diluted 10-fold. The dilutions were spread plated (100 µl) onto tryptone soya agar. The plates were incubated aerobically at 30°C for 48 h. Colonies were isolated randomly based on the differences in the morphology and the cell-free culture supernatant (CFCS) of the isolated colonies were screened for antibacterial activity against *E. coli* ATCC 25922 by well diffusion assay.

Preparation of cell free culture supernatant (CFCS)

For preparation of CFCS, a single colony of soil isolates was inoculated into 5 ml tryptone soya (TSB) broth (w/o dextrose) and incubated at 30°C, 200 rpm for 24 to 48 h until saturated growth was observed. This saturated culture was subcultured (1%) into fresh 10 ml TSB (w/o dextrose) and incubated at 30°C, 200 rpm. After 12 h, 24 h, 48 h, and 72 h of incubation, 1 ml culture was withdrawn, centrifuged at 12,000 rpm, 4°C for 15 min and the supernatant was filtered through 0.22 µm filter (Millipore) to obtain CFCS.

Well diffusion assay

Well diffusion assay was done as described earlier (Schillinger and Lücke, 1989) with some modifications. Briefly, the CFCS (100 µl) of soil isolates was added in wells on Mueller Hinton agar (MH; Merck) plates (for testing activity against bacteria) or YEPD plates for testing activity against yeast) previously spread with 100 µl (105 CFU/ml) suspension of indicator strain present in its logarithmic phase of growth. The plates were incubated for 12 h at 37°C. The experiment was done in duplicates. A positive control [Chloramphenicol (Sigma)] for bacterial strains and Amphotericin B [(Sigma) for yeast] was included along with a negative control (culture media without any microbial growth). Zone of inhibition of antibacterial agents tested was measured after incubation. Inhibition of bacteria was scored positive if the zone was wider than 1 mm.

Antimicrobial susceptibility testing by broth microdilution method

In broth microdilution method the, antibacterial agent was twofold serially diluted in MH broth (Merck) in 96-well

Table 1. Spectrum of activity of RPT-0001

Indicator strain	AU/ml[a]
Gram-negative	
E. coli ATCC 25922	80
E. coli MC 1061 MTCC946	1280
E. coli DH5α MTCC483	160
Shigella flexneri ATCC9199	640
Chronobacter sakazakii MTCC659	40
Salmonella enterica subsp. *enterica* ATCC 10708	40
Gram-positive	
Listeria monocytogenes MTCC657	320
Clostridium perfringens MTCC450	640
Staphylococcus aureus ATCC29213	640
Bacillus subtilis MTCC441	160
Bacillus cereus ATCC 11778	80
Micrococcus luteus ATCC 10240	2560
Yeast	
Candida albicans ATCC 10231	No inhibition[b]

[a] Arbitrary units per milliliter (AU/ml) is defined as the reciprocal of the highest dilution of CFCS completely inhibiting bacterial growth.
[b] The inhibitory activity of RPT-0001 CFCS against *C. albicans* ATCC 10231 was determined by well diffusion assay. No inhibition indicates absence of zone of inhibition.

plates (Genaxy) and approximately 10^5 bacterial cells (cultured in MH broth) in their log phase of growth were added to each dilution of the antibacterial agent. The inoculum size of the test isolates was standardized according to the Clinical and Laboratory Standards Institute (CLSI, 2009). A positive control for bacterial growth (cells without any antibacterial agent) and negative control (uninoculated media) were also included. The plates were incubated at 37°C without shaking and the growth was monitored by optical density at 600 nm (OD_{600}) readings after 12 h of incubation using Spectramax plus plate reader (Molecular devices).

Antimicrobial activity of CFCS was expressed as Arbitrary units per milliliter (AU/ml) and defined as the reciprocal of the highest dilution completely inhibiting bacterial growth after 12 h of incubation and calculated according to formula $AU/ml = [(D^n \times 1{,}000)/V]$, where D = the dilution factor; n = number of the highest dilution showing complete bacterial inhibition; V = volume of the CFCS used in first well. The bacterial indicator strains used are documented in Table 1.

Time dependent kill kinetics of RPT-0001 crude extract

Time dependent kill kinetic assay of RPT-0001 was evaluated as described previously (Ling *et al*., 2015) with some modifications. Briefly, a single colony of *E. coli* MC1061 was inoculated from a fresh MH-agar plate into 5 ml MH broth and incubated overnight at 37°C with agitation of 200 rpm. 1% of overnight grown saturated culture was again subcultured into fresh MH broth and incubated at 37°C with same conditions until the OD_{600} of the culture reaches at 0.5. This culture was diluted 1,000 times in fresh MH Broth in three different tubes. One tube was kept as control without adding any antibacterial extract and in other two tubes 2× and 4× volume of RPT-0001 crude extract was added. All three tubes were incubated at 37°C at 200 rpm. 100 µl of aliquots were withdrawn at an interval of 2 h from each tube, diluted appropriately in sterile 1X PBS and plated on MH-agar plate in triplicates. The plates were incubated at 37°C overnight. The number of colonies on each plate was counted and average number of cells in CFU/ml was estimated. The changes in the number of cells at three different conditions were plotted against time.

Identification and phylogenetic analysis of the bacterial isolate producing antibacterial activity

The antibacterial strain isolated from soil was identified according to its physiological and biochemical characteristics namely Gram staining, endospore staining, carbohydrate utilization, citrate utilization, esculin hydrolysis, starch hydrolysis, casein hydrolysis and catalase reaction. KB009 Hi-Carbohydrate kit (Himedia) was used for carbohydrate utilization tests. In addition to biochemical tests, biomolecular identification was performed. Genomic DNA of the antibacterial isolate was prepared. A pair of primers specific for bacterial 16S rDNA, 8-27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) were used to amplify the corresponding gene. Amplification by PCR involved using a Hot start taq DNA polymerase (Qiagen) under the following conditions: after an initial 3-min incubation at 95°C, the mixture was subjected to 30 cycles, each including 1 min at 95°C, 30 sec at 52°C, and 2 min at 72°C. A final extension was performed at 72°C for 10 min. The amplified 16S rDNA was purified using a commercial gel extraction kit (QIAquick gel extraction kit; Qiagen), and sent for sequencing at Ocimum biosolutions, India. The derived 16S rDNA gene sequence (1.5 kb) was compared with known bacterial sequences in the National Center for Biotechnology Information (NCBI) GenBank using Basic Local Alignment Search Tool (BLAST). Only results from the highest score queries were considered for phylotype identification, with 98% minimum similarity. The phylogenetic analysis was performed using twenty two 16S rDNA sequences of bacteria closely related to *Bacillus* sp. RPT-0001 by Juke and Cantor nucleotide substitution model for sequence alignment, with 1,000 iterations of bootstrapping. The phylogenetic tree was constructed by PHYLIP 3.69 (http://evolution.genetics.washington.edu/phylip.html) and visualized using TreeViewX.

Effect of enzymes on antibacterial activity of RPT-0001

The sensitivity of the CFCS and purified antibacterial fraction towards different proteases was evaluated. Different hydrolytic enzymes including trypsin, chymotrypsin, proteinase K and pronase E at a final concentration of 5 mg/ml were incubated with CFCS and purified antibacterial fraction for 3 h at 37°C and checked for antibacterial activity by broth microdilution method. In all the above mentioned studies, uninoculated medium TSB (w/o dextrose) was exposed to the same conditions (digestive enzymes, pH, and temperature) as were the CFCS or the purified fraction and used as negative control against *E. coli* ATCC 25922 and *B. cereus* ATCC 11778. The experiment was carried out in triplicates.

Table 2. **Purification stages of RPT-0001 and activity against *E. coli* ATCC 25922 determined by broth microdilution assay**

Purification stage	Volume (ml)	AU/ml
CFCS	500	80
1 kDa permeate concentrated by lyophilization	0.5	2560
HPLC eluate lyophilized and dissolved in water	0.1	5120

Growth profiling and antibacterial peptide production by the antibacterial isolate

Production of the antimicrobial substance by *Bacillus* sp. RPT-0001 was determined at different phases of growth by plotting growth versus antibacterial activity. For preparation of growth curves, 3 flasks for antibacterial activity producing strain were prepared with TSB (w/o dextrose) medium and inoculated with 1% (v/v) of log phase bacterial culture (*Bacillus* sp. RPT-0001). One flask was kept uninoculated as negative control. The flasks were incubated in shaking incubator at 30°C and 200 rpm. After every hour, the optical density OD_{600} was noted using Spectramax plus plate reader (Molecular devices). Each sample after every hour was also checked for inhibitory activity against *E. coli* ATCC 25922 using broth microdilution method.

Bioassay-guided fractionation to purify RPT-0001

The crude antibacterial CFCS obtained after 16–20 h growth was purified and tested for antimicrobial activity against *E. coli* ATCC 25922 and *B. cereus* ATCC 11778 at every step of purification using well diffusion assay. To remove large proteins, the CFCS (500 ml) was passed sequentially through macrosep 10 kDa, 3 kDa, and 1 kDa cutoff centrifugal devices (Pall Corporation). The 1 kDa permeate was lyophilized and resuspended in 500 µl H_2O (MilliQ). This was followed by separation and purification by HPLC (Waters 600 analytical HPLC system, Milford). A 20 µl sample was applied to C-18 reverse phase column (4.6 × 100 mm, pore size 80 A°) pre-equilibrated with methanol and water. The mobile phase consisted of (i) HPLC-grade methonal (Merck) and (ii) HPLC-grade water containing 0.1% trifluoroacetic acid (Sigma). A gradient elution of 20% to 100% methanol was used for separation. The flow rate was 1 ml/min. Elution was monitored at a wavelength of 220 nm, and fractions (corresponding to major peaks) were collected manually for the antimicrobial activity bioassay. The entire process was repeated 20 times. Fractions collected in separate runs which had same retention time were pooled, lyophilized, dissolved in 100 µl water and screened for antibacterial activity in a well diffusion assay. The purified fraction with antibacterial activity was re-injected in same column under same conditions to check purity of eluted fraction. The antibacterial activity in terms of AU/ml was also calculated against *E. coli* ATCC 25922 at each stage of purification and concentration (Table 2) by broth microdilution assay.

Electron spray ionization-mass spectrometry (ESI-MS) of RPT-0001

HPLC purified RPT-0001 antibacterial fraction was extracted 3 times with one-fourth volume of butanol by vortexing for 20 sec followed by 2 min centrifugation at 13,000 rpm to separate the organic phase. Collected butanol layer was evaporated to dryness by lyophilisation. The residue was dissolved in 10% methanol and analyzed with Electrospray Ionization Mass Spectrometer (ESI-MS; Bruker daltonics) in positive ion mode.

Synthesis and characterization of RPT-0001 capped silver nanoparticles (SNPs)

RPT-0001 capped SNPs were prepared by addition of freshly prepared silver nitrate (1 mM) to RPT-0001 purified peptide dilutions in miliQ water. The resultant mixture was incubated for 0–40 min time periods under illumination at 30°C under 40 W yellow light (141.3 lux). The optimum RPT-0001 concentration for this reaction was determined by a set of batch experiments where the concentration of the peptide was varied between 0 and 100 µg/ml. After half an hour, the colour of the solution changed from colourless to yellow, indicating the formation of silver nanoparticles. The prepared RPT-001 capped SNPs were pelleted down by centrifuging the sample at 20,000 rpm for 20 min and were washed twice with miliQ water to remove unbound silver nitrate and RPT-001 peptide. Citrate-capped silver nanoparticles (CSNPs) were synthesized at 30°C using successive addition of silver nitrate (1 mM), tri sodium citrate (0.15 mM), sodium borohydride (0.6 mM) and were used as control in experiments. The prepared SNPs (RPT-0001 capped SNPs and CSNPs) were characterized by UV-visible spectroscopy (Molecular devices). The concentration of silver ions (Ag^+) in RPT-0001 capped SNPs and CSNPs was determined by Atomic Absorption Spectrophotometer (AAS, GBC Scientific Equipment). The morphology and size of nanoparticles were observed using atomic force microscopy (AFM; NTEGRE, NT-MDT) and scanning electron microscopy (SEM; Carl Zeiss).

Determination of minimum inhibitory concentration (MIC) and fraction inhibitory concentration (FIC)

Antimicrobial activity of synthesized SNPs (RPT-0001 capped SNPs and CSNPs) was expressed in terms of MIC (minimum inhibitory concentration). MIC was deduced as the lowest concentration of SNPs at which the growth of the tested indicator strains was inhibited completely after 12 h of incubation. The experiment for determination of MIC of RPT-0001 capped SNPs and CSNPs was carried out in triplicates. The synthesized SNPs were tested for antibacterial activity against food-borne bacterial pathogens by broth microdilution method and the experiment was performed in triplicates.

To determine whether CSNPs (compound A) and RPT-001 (compound B) worked synergistically or additively, the FIC index was used. The FIC index was calculated against *E. coli* MC1061 as using following equation:

$$\frac{\text{MIC of compound A in combination}}{\text{MIC of compound A alone}} + \frac{\text{MIC of compound B in combination}}{\text{MIC of compound B alone}}$$

FIC index values lower than 0.5 indicate synergistic activity, values in between 0.5 and 2.0 indicate additive effects, whereas values above 2.0 indicate antagonistic effects (Ruden *et al.*, 2009).

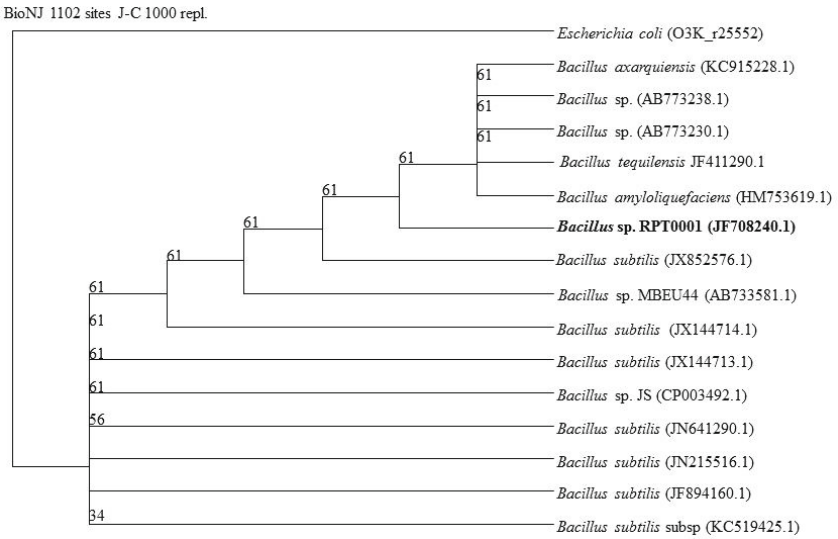

Fig. 1. Phylogenetic tree based on 16S rRNA nucleotide sequences. A neighbour-joining analysis with Jukes-Cantor correction and bootstrap support was performed on the gene sequences. The number at the nodes indicates the level of bootstrap support on neighbour-joining analysis of 1,000 resampled data set. Values in parentheses are accession numbers.

Results

Isolation and identification of an antibacterial strain from soil and its phylogenetic analysis

Cell free culture supernatants of a large number (221) of soil isolates in tryptone soya broth (w/o dextrose) were screened for antimicrobial activity against *E. coli* ATCC 25922. One of the isolate showed promising antibacterial activity and was further screened for activity against food-borne bacterial pathogens and was found to be inhibitory (Table 1). Specific biochemical, morphological and 16S rRNA sequencing results (data not shown) revealed that the isolate belongs to Genus *Bacillus* and was designated as *Bacillus* sp. RPT-0001. The isolate was found to be taxonomically close to *B. subtilis*. *Bacillus* sp. RPT-0001 16S rRNA gene partial sequence was submitted to NCBI under Accession No.JF708240. A phylogenetic tree was constructed as outlined in 'Materials and Methods' and it is clearly indicated from the tree that the identified bacterium is closely related to *Bacillus* genus (Fig. 1).

Characterization of antibacterial biomolecule RPT-0001

The antibacterial biomolecule RPT-0001 produced by soil isolate *Bacillus* sp. RPT-0001 presented a large spectrum of activity, inhibiting the growth of food spoilage bacteria and food-borne pathogens, including *L. monocytogenes*, *C. perfringens*, *B. cereus*, *S. aureus*, *C. sakazakii*, *Salmonella enterica* subsp. *enterica* ATCC 10708, *Shigella flexneri*, and *E. coli*. However, no inhibitory activity was detected against the *C. albicans* ATCC 10231 (Table 1). Treatment of the CFCS with proteolytic enzyme pronase E (5 mg/ml) for 3 h resulted in significant reduction of antimicrobial activity against *E. coli* (Fig. 2). Similar results were obtained for *B. cereus* (data not shown) indicating that the antibacterial molecule bears a peptide as an antibacterial moiety. Antibacterial activity of RPT-0001 CFCS was tested by broth microdilution method after passing sequentially through centrifugal membranes of 10 kDa, 3 kDa, and 1 kDa cutoff size. As the antibacterial activity was observed in permeates of all the cutoff membranes tested (data not shown), it is apparent that the size of RPT-0001 antibacterial peptide is less than 1 kDa.

The small antibacterial peptide was purified by passing through various cutoff centrifugal devices followed by RP-HPLC. The AU/ml of the antibacterial fraction increased at every step of purification and concentration (Table 2). The intact mass of HPLC eluted antibacterial fraction was analyzed by ESI-MS, which also confirmed the purity of the fraction. The result of ESI-MS analysis revealed that the molecular mass of the purified antibacterial peptide from *Bacillus* sp. RPT-0001 is 276.9 Da which indicates that it may be a dipeptide (Fig. 3).

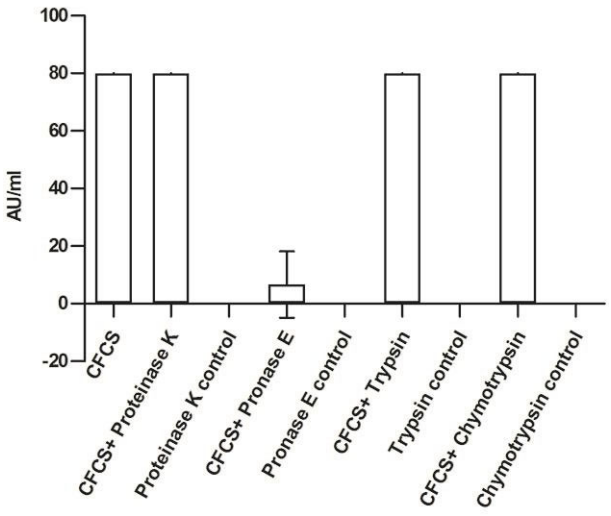

Fig. 2. Activity of proteolytic enzyme treated RPT-0001 cell free culture supernatant (CFCS) against *E. coli* ATCC 25922. The concentration of enzyme used was 5 mg/ml. Incubation of enzyme with CFCS was done at 37°C for 3 h. Enzyme control indicates TSB (w/o dextrose) alone treated with same concentration of enzyme as used for treating CFCS and incubated under same conditions (pH 7.2, 37°C for 3 h).

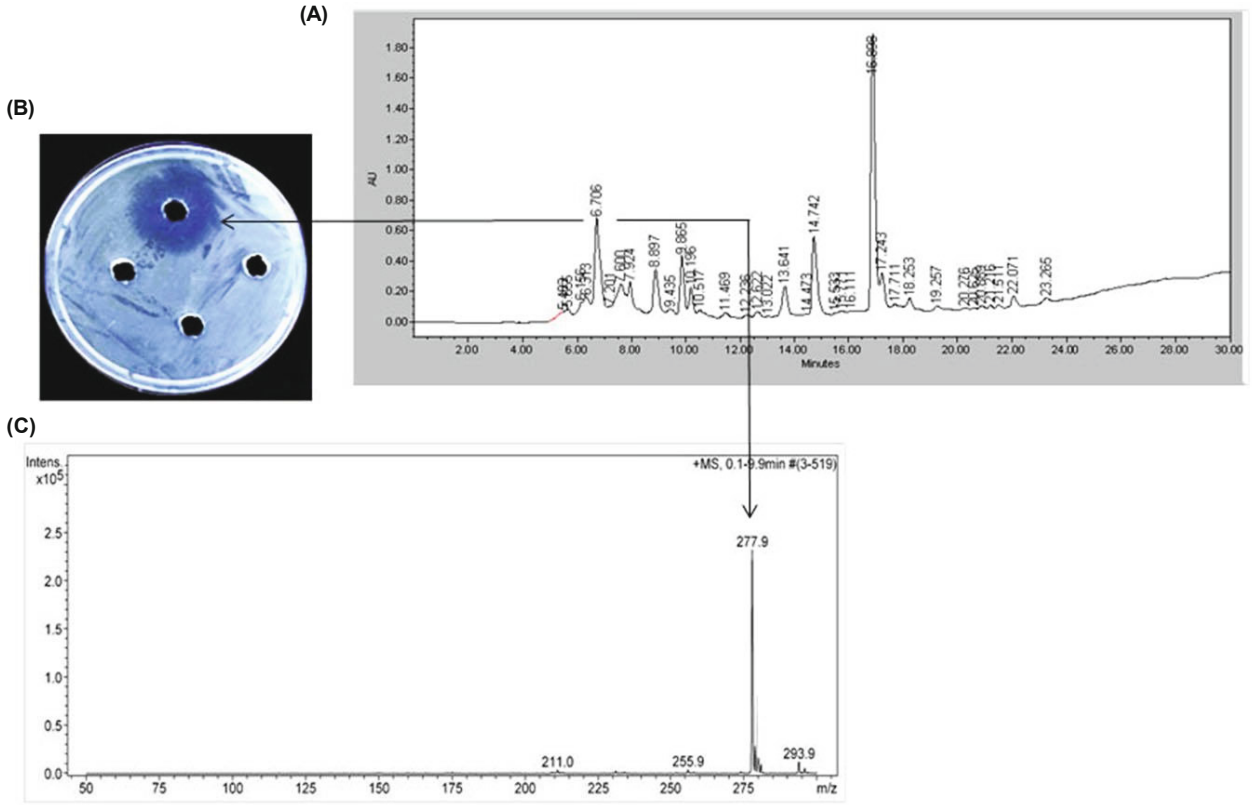

Fig. 3. Reverse-phase high-performance liquid chromatography (RP-HPLC) chromatogram and electospray ionization (ESI) mass spectrometry (MS) data of RPT-0001. (A) HPLC profile of *Bacillus* sp. RPT-0001 CFCS in TSB (w/o dextrose) after passing through 1 kDa membrane. Elution was done in 20 to 100% methanol gradient and detection was done at 220 nm wavelength. (B) Well diffusion assay plate of the HPLC fraction inhibitory to *E. coli* ATCC 25922. (C) Electrospray ionization mass spectrum of fraction eluted from HPLC (C_{18}) column showing antibacterial activity in well diffusion assay.

Growth dynamics of *Bacillus* sp. RPT-0001 and antibacterial peptide production

Bacillus sp. RPT-0001 produced extracellular inhibitory activity against *E. coli* ATCC 25922 in tryptone soya broth (w/o dextrose) when incubated at 30°C with 200 rpm shaking. The CFCS collected at regular time intervals during bacterial growth was used to perform antimicrobial activity assays against *E. coli* ATCC 25922. The results showed that RPT-0001 production started after 9 h of growth. However, there was a significant increase in production between 14 to 22 h and the antibacterial activity decreased thereafter.

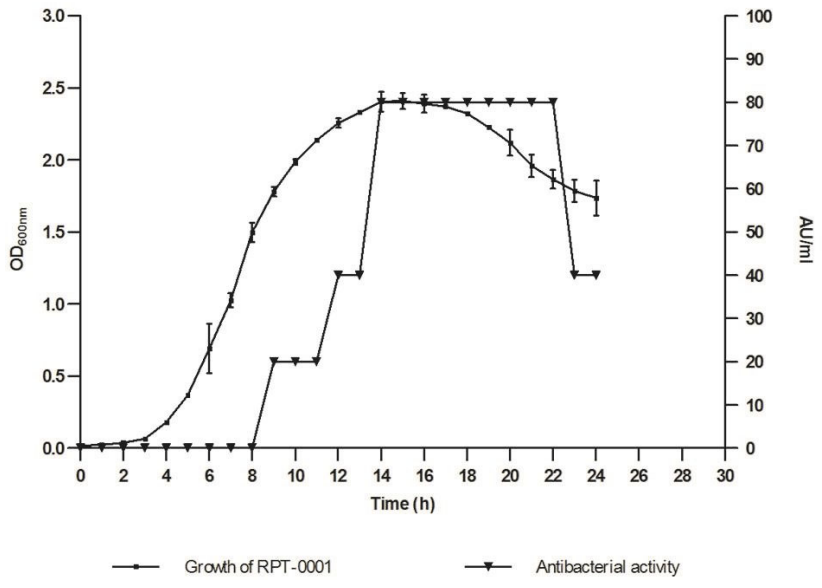

Fig. 4. Comparison of *E. coli* inhibitory activity production in different phases of *Bacillus* sp. RPT-0001 growth. The growth curve analysis of *Bacillus* sp. RPT-0001 indicates that the production of the antibacterial agent initiated at early logarithmic phase. Growth measured as absorption at 600 nm is indicated by circles (left y axes), while the antibacterial activity is indicated by squares (right y axes). The activity was measured against *E. coli* ATCC 25922.

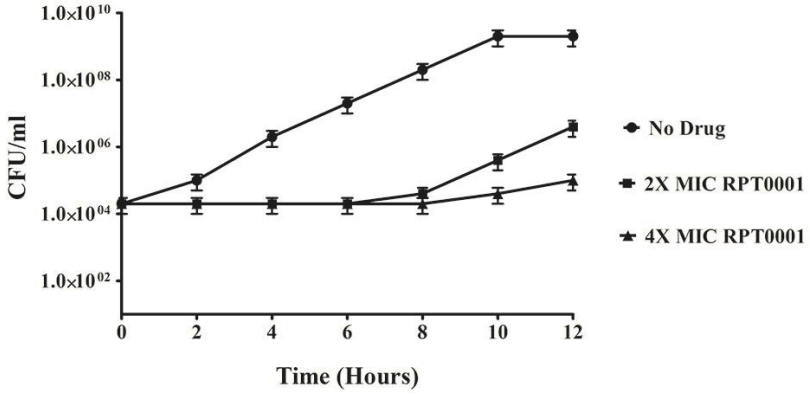

Fig. 5. Time dependent kill kinetic of RPT0001 crude extract. Kill kinetics of RPT0001 crude extract was determined against *E. coli* MC1061. Approximately 10^5 CFU/ml cells of *E. coli* MC1061 were challenged with 2× and 4× MIC of RPT0001 crude extract and growth were monitored up to 12 h with time interval of 2 h. In the control tube no antibacterial extract was added.

Production of antibacterial activity started at early exponential growth phase and the maximum production was in stationary phase indicating that RPT-0001 is likely a secondary bacterial metabolite (Fig. 4).

Determining the time dependent kill kinetics of RPT-0001

Time kill kinetics of RPT-0001 crude extract against *E. coli* MC1061 was determined. Approximately 10^5 CFU/ml *E. coli* cells were challenged with 2× and 4× MIC volumes of RPT0001active crude extract and growth was monitored up to 12 h with time interval of 2 h. The initial number of cells remained almost constant up to around 6 and 8 h for 2× and 4× MIC, respectively. After 6–8 h a gradual increase in the cell numbers were observed. The revival of the cells in the static condition is much slower than this. No visible growth was observed upto 24 h at 2× and 4× MIC in static conditions.

RPT-0001 mediated antibacterial silver nanoparticle synthesis

Synthesis of RPT-0001 was optimized by varying concentrations of RPT-0001 (0–100 μg/ml) and a fixed concentration of silver nitrate (1 mM) with different incubation times (0–40 min). The silver nanoparticles formation was confirmed by the color change of silver nitrate solution from colorless to yellow in 30 min and further confirmed with the help of UV-visible (UV-vis) spectroscopy. The UV-vis absorption spectrum of the resulting solution displayed characteristic surface plasmon resonance band of spherical SNPs centered around 420 nm (Fig. 6). For CSNPs, a characteristic SPR peak was observed at 420 nm (Fig. 6). Scanning electron microscopy (SEM) analysis revealed that shape of CSNPs was spherical with diameter size of 15–20 nm (Fig. 7A). However, capping of RPT-0001 to SNPs increased the size of nanoparticles which was observed to be 20–30 nm using SEM (Fig. 7B) and atomic force microscopy (AFM) analysis (Fig. 7C, D, and E). RPT-0001 probably played the role of capping agent in the synthesis of SNPs as no other capping agent was provided. For the rest of the experiments, a solution consisting of 1 mM silver nitrate and 100 μg/ml of RPT-0001 in miliQ water and an incubation time of 30 min was taken for nanoparticles synthesis. The synthesized RPT-0001 capped SNPs clearly showed decrease in MIC (2 to 8 fold) as compared with CSNPs for their antibacterial activity against both Gram-negative and Gram-positive food-borne pathogens (Table 3). As described in 'Materials and Methods' section, the FIC index was used to investigate the synergistic, additive, or antagonistic effect between CSNPs and RPT-0001 against *E. coli* MC1061. The FIC index was 0.129 against *E. coli* MC1061 indicating that capping of RPT-0001 to the SNPs produced the synergistic antimicrobial effect.

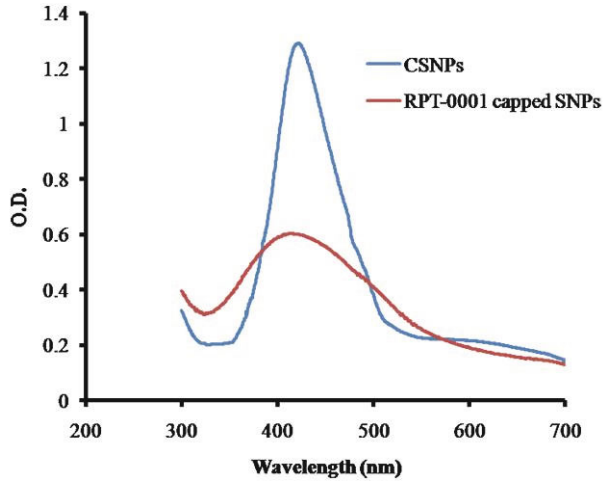

Fig. 6. UV-visible spectra of CSNPs and RPT-0001 capped SNPs. UV-vis absorption spectrum displayed characteristic surface plasmon resonance band of spherical SNPs centered around 420 nm.

Table 3. Comparison of MIC of RPT-001 SNPs with citrate capped SNPs against various pathogens

Indicator strain	MIC[a] (μg/ml)	
	Citrate capped SNPs	RPT-001 SNPs
E. coli ATCC 25922	12.5	1.6
E. coli MC1061 MTCC946[b]	0.85	0.11
Chronobacter sakazakii MTCC659	25	3.2
Salmonella enterica subsp. *enterica* ATCC10708	3	1.6
Listeria monocytogenes MTCC657	25	6.4

[a] MIC (minimum inhibitory concentration) is defined as highest dilution of SNPs inhibiting growth of indicator bacteria completely.
[b] MIC value of RPT-0001 for *E. coli* MC1061 was 3.80 mg/ml. This value and the corresponding MIC value of CSNP and RPT-0001 SNPs were used for calculating FIC index.

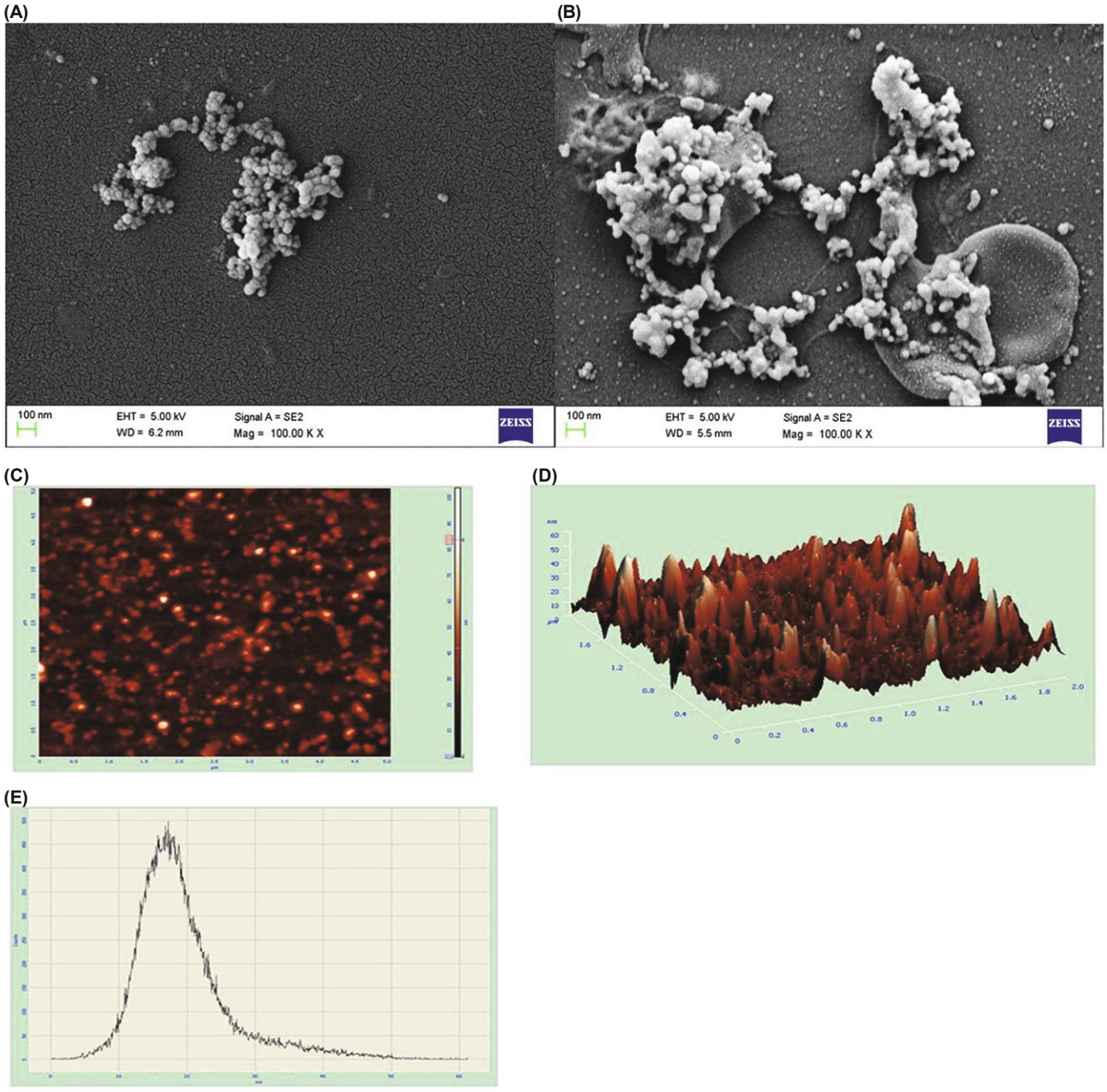

Fig. 7. Scanning electron micrograph and atomic force micrograph (AFM) of CSNPs RPT-0001 capped SNPs. (A) SEM of CSNPs (B) SEM of RPT-0001 capped SNPs (C) AFM scan of SNPs synthesized using RPT-0001 as a capping agent showing spherical morphology of SNPs. (D) 3D (three dimensional) AFM scan (E) AFM size distribution histogram indicating average size of nanoparticles.

Discussion

Food-borne diseases ensuing from consumption of food contaminated with pathogenic bacteria and/or their toxins is a significant concern to public health (Kim *et al.*, 1995). The spread of antibiotic resistant bacteria in food and the consumer preference for food materials with reduced or no chemical preservatives has stimulated the search for natural antimicrobial agents (Gálvez *et al.*, 2008; Baruzzi *et al.*, 2011). The genus *Bacillus* consists of Gram-positive, facultative or aerobic endospore forming bacilli known to produce a large number of bioactive secondary metabolites including antibacterial peptides (Abriouel *et al.*, 2011; Baruzzi *et al.*, 2011). Antibacterial peptides from *Bacillus* spp. are useful because of their broader spectrum of inhibition (as compared with antibacterial peptides produced by most lactic acid bacteria), which may include Gram-negative bacteria, yeasts or fungi (Abriouel *et al.*, 2011). The GRAS status of most of *Bacillus* species and the ability to produce inhibitory compounds, mainly active against food-borne pathogens indicate that these metabolites could be applied in food products to reduce the level of pathogens (Baruzzi *et al.*, 2011).

A bacterial isolate from soil sample presenting antimicrobial activity indicated 99% identity to *B. subtilis* based on 16S rRNA gene sequencing. It was designated as *Bacillus* sp. RPT-0001. The CFCS of *Bacillus* sp. RPT-0001 was able to inhibit the growth of many food spoilage bacteria and food-borne pathogens, including *L. monocytogenes*, *C. perfringens*, *S. aureus*, *B. subtilis*, *B. cereus* ATCC 11778, *M. luteus* ATCC 10240, *C. sakazakii*, *S. enterica* subsp. *enterica* ATCC 10708, *S. flexneri* ATCC 9199, and various species of *E. coli*. The antibacterial activity of CFCS was deactivated in the presence of proteolytic enzyme pronase E within 3 h of treatment at a concentration of 5 mg/ml. However the activity was resistant to proteinase K, trypsin and chymotrypsin treatment. Similar results were observed when purified antibacterial fraction was tested. RPT-0001 antibacterial peptide was purified by passing sequentially through 10 kDa, 3 kDa, and 1 kDa cutoff membranes followed by reverse phase HPLC and the molecular mass was determined to be 276.9 Da by ESI-MS. These results were compared with known antibacterial peptides (bacteriocins, bacteriocin-like inhibitory substances and non-ribosomally synthesized antibacterial peptides) from *B. subtilis* and other species of *Bacillus* (Tagg *et al.*, 1976; Zheng and Slavik, 1999; Le Marrec *et al.*, 2000; Kawulka *et al.*, 2004; Stein, 2005; Teo and Tan, 2005; Abriouel *et al.*, 2011; Baruzzi *et al.*, 2011; Lee, 2011). No match was found, indicating the possible discovery of a novel antibacterial peptide.

Further, synthesis of silver nanoparticles was carried out using RPT-0001 as a capping and reducing agent and its activity on food-borne bacterial pathogens was tested. Although nanoparticles can be made using diverse physicochemical methods, their synthesis by means of nontoxic biological methods is attractive particularly if they are projected for applications in food or medicine. Biomolecules present in bacterial extracts can be used to reduce metal ions to nanoparticles in a single-step green synthesis process (Hebbalalu *et al.*, 2013). Extracts of a diverse range of bacterial species have been successfully used in making nanoparticles (Samadi *et al.*, 2009; Ramanathan *et al.*, 2010; Debabov *et al.*, 2013; Malarkodi *et al.*, 2013; Paulkumar *et al.*, 2013). The RPT-0001 synthesized silver nanoparticles were inhibitory against all food-borne bacterial pathogens tested implicating their potential in various antibacterial applications involving food-borne pathogens. Moreover, as no capping agent was used, RPT-0001 probably played the role of a capping agent as well. However, further experiments are needed to confirm this. Silver nanoparticles were rapidly synthesized using RPT-0001 purified fraction and the formation of nanoparticles was observed within 1 h. The results recorded from UV-vis spectrum, SEM and AFM support the biosynthesis and characterization of silver nanoparticles. The synthesized silver nanoparticles showed effective inhibitory activity against food-borne pathogens viz., *L. monocytogenes*, *C. sakazakii, S. enterica* subsp. *enterica*, and *E. coli*. RPT-0001 showed excellent antibacterial synergy with SNPs as compared with CSNPs.

This study shows isolation and partial characterization of a novel antibacterial peptide from soil bacilli, green and facile synthesis of antibacterial peptide capped silver nanoparticles holding potential application against food-borne pathogens. The antibacterial peptide displays characteristics which do not match other antibacterial peptides produced by *Bacillus* sp. and related genera. The peptide RPT-0001 did not show any toxicity to eukaryotic model organism *C. albicans*. The overall results of this study can be considered as promising in the perspective of new drug discovery from bacterial sources and suggest the feasibility of producing antibacterial SNPs from antibacterial peptide effective against food-borne bacterial pathogens. These coated SNPs can be effectively used for developing antimicrobial packaging to extend shelf life of perishable foods. Their direct addition to the foods will still warrant detailed toxic studies.

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