Nature Environment and Pollution Technology *An International Quarterly Scientific Journal*	ISSN: 0972-6268	Vol. 12	No. 2	pp. 209-214	2013

Original Research Paper

Study on Bioactive Compounds Produced by Soil Origin *Brevibacillus* spp.

S. Seyed Gholizadeh, M. Baserisalehi* and N. Bahador
Department of Microbiology, Science and Research Branch, Islamic Azad University, Fars, Iran
*Department of Microbiology, Kazerun Branch, Islamic Azad University, Kazerun, Iran

Nat. Env. & Poll. Tech.
Website: www.neptjournal.com

Received: 10-1-2013
Accepted: 20-2-2013

Key Words:
Brevibacillus spp.
16SrRNA sequencing
Antimicrobial effect
Bioactive compounds
Probiotics

ABSTRACT

The purpose of this study was the evaluation of soil origin *Brevibacillus* for production of bioactive compounds. Nowadays bioactive compounds are considered the sources of new remedy in order to eliminate antibiotic-resistant bacteria. To perform the study, two strains of *Brevibacillus* with potent activity for production of bioactive compounds were isolated from 121 soil samples. Phenotypic and 16SrRNA sequencing identification of the isolates recognized them as *Brevibacillus reuszeri* (B12 bioactive compound producer) and *Brevibacillus formosus* (B22 bioactive compound producer). The bioactive compounds produced by the bacteria were partially purified and characterized based on their antimicrobial activities at various pHs and temperatures and proteinase K. Then, activity of the bioactive compounds was assessed against different pathogenic microorganisms. The results obtained indicated that activity of the compounds when treated with proteinase K was inhibited. The optimum pH for both the isolates was 7 and potent activity of the bioactive compound B12 was observed at 35°C while for B22 was at 40°C. Although, *Bacillus cereus, Staphylococcua aureus, E. coli* and *Salmonella typhi* were sensitive, *Pseudomonas aeruginosa* and fungi (*Candida albicans* and *Aspergillus niger)* were resistant to the both bioactive compounds. Therefore, *Brevibacillus* with potent activity for production of antimicrobial metabolites, and fast growth character may be considered as a good probiotic agent for commercial industries.

INTRODUCTION

Bioactive compounds are a group of compounds produced by microorganisms with antimicrobial property. Antibiotics and bacteriocins are bioactive compounds with different antimicrobial spectrum and property. These compounds differ from each other based on their production. Bacteriocins are produced in the exponential phase while, antibiotics are produced in the stationary phase. Therefore, bacteriocins are primary metabolites, whereas antibiotics are secondary metabolites (Oscariz & Pisabarro 2001).

Nowadays high frequency of occurrence of antibiotic resistant bacteria culminates in serious attention on production of another bioactive compound such as bacteriocins. In addition, preservation of food with safe compounds is also another demand to reduce the rate of antibiotic resistant strains of pathogenic bacteria, and in this regard production of bioactive compounds by probiotics is a major field of investigation (Gillor et al. 2005).

Although lactic acid bacteria are most famous probiotic microorganisms, spore forming bacteria have also been studied as probiotics (Sanders et al. 2003).

Brevibacillus species is spore forming bacteria living in the soil. This genus previously was belonging to the genus *Bacillus* and reclassified based on their gene sequence analyses in 1996 (Shida et al. 1996). However, *Brevibacillus brevis* was studied for its biocontrol property against plant pathogens (Edwards & Seddon 2001), but less information is available concerning to their ability for production of bioactive compounds.

Therefore, in order to achieve information concerning to production of bioactive compounds by *Brevibacillus,* the present study was conducted to isolate and identify the bacteria from soil and their evaluation for production of bioactive compounds.

MATERIALS AND METHODS

Sample collection: Hundred and twenty one soil samples were collected from five locations in Shiraz in southern Iran.

Isolation of spore forming bacteria from soil was carried out by serial dilution of soil sample (10^{-1}- 10^{-7}), followed by heating at 80°C for 10min. Then, 0.1mL of each dilution was transferred onto Brain Heart infusion agar and the plates were incubated at 35°C for 24h. Following the observation of colonies, each colony was picked up and subjected to bacteriocin production assay.

Screening of bioactive compound producing bacteria: The soil samples were diluted from 10^{-1} to 10^{-7}. Then, 0.1 mL from 10^{-4} - 10^{-7} dilution was cultured on the Brain Heart Infusion agar. The plates were incubated at 35°C for 24 hours. Following the incubation, the bacterial colonies were purified and subjected to the production of bacteriocins using well-diffusion agar method.

To perform the experiment the bacterial colonies were separately inoculated into hundred milliliters of trypticase soy broth in conical flask (250 mL) and incubated at 35°C, 200 rpm for 24h. Then, 5mL of each culture broth was centrifuged at 12000 rpm for 30 min (Baserisalehi & Bahador 2012) and the supernatant was assessed for antimicrobial property against bioassay strains of bacteria viz., *E. coli* PTCC 1330 and *Staphylococcus aureus,* PTCC 1337.

Screening of bacteriocin producing strains was done by Well Diffusion Agar (WDA) method. The experiment was carried out by cultivation of the bioassay strain on Mueller Hinton agar and then the wells (5 mm in diameter) were made in plate agar using sterile sharp borer. After that, 100 µL of each supernatant was added to the each well and the plates were incubated at 35°C for 24h. Screening of the promising strain for production of bacteriocin was carried out by observation of growth inhibition zone around the well filled with the bacterial metabolite.

Identifications of bioactive compound producing bacterial strains: Bioactive compounds producing bacteria were presumptively identified by microscopic and macroscopic analysis viz., Gram stain and colony morphology, respectively. Then, biochemical tests such as O/F, Nitrate, TSI and Urease, were done based on Bergey's Manual of Systematic Bacteriology (Garrity 2009). Authentication of the bioactive strains was done by 16SrRNA gene DNA sequencing. To perform the experiment DNA extraction was carried out using DNA PCR kit (Roche-Germany). Then the purity of extracted DNA was assessed by absorbance at 260 and 280 nm. The extracted DNA with ratio (260/280nm) of 1.9≤ corresponding to 121µg DNA per mL was used for Polymerase Chain Reaction (PCR). Amplification of 16SrRNA gene was performed using Forward and Reverse primers with sequences of 5'-CAACGAGCGCAACCCT-3'and 5'-GGTTACCTTGTTACGACTT-3' respectively. Each reaction tube was containing 14.5 µL of water (Sigma Aldrich Company Ltd.), 2.5 µL of 10 × PCR buffer (Cinagen-Iran), 1 µL of each forward and reverse PCR primers, 1 µL of a 10 mM dNTPs (Cinagen-Iran), 0.5 µL of Smar taq polymerase (cinagen-Iran), 1 µL of 50mM $MgCl_2$ (Cinagen-Iran) and 5 µL of DNA template. PCR conditions of thermocycler (Cleaver, England) were as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 60s, 56°C for 45s, and 72°C for 60 s, with a final extension at 72°C for 5 min and storage at 4°C. All the PCR products were run on a 1.5% (w/v) agarose gel. PCR products were electrophoresed at 75V for 20 min and then DNA bands were virtualized after staining with ethidium bromide. Finally the PCR products with pure DNA bands have been sent to Macrogen in South Korea (http://www.macrogen.com/) for DNA sequencing. The 16S rRNA sequenced data were subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) to identify each respective 16S rRNA gene amplicon.

Partial purification of bioactive compounds: Ammonium sulphate (80% saturation) was added to the culture supernatants and the tubes were kept on ice for 18-24 h. Then precipitated proteins were collected by centrifugation (10,000 rpm, 30 min) and suspended in phosphate buffer solution (PBS, pH 6.8) and Catalase (2 mg/mL) was added into the solution and centrifuged for 15 min at 8000rpm. Afterward 50 mM sodium phosphate buffer (pH 6.5) was added to the mixture, treated with chloroform-methanol (2:1, v/v) for 1 h and then centrifuged at 12000rpm, 30 min, 4°C . The precipitate was air dried and dissolved in 10 mL solution of 10 % 2-propanol and 0.1% trifluro acetic acid (TFA) (Baserisalehi & Bahador 2012).

Growth phase of bioactive compound production: Production of antimicrobial compounds at different time intervals of 5, 10, 15, 20........30 h was assessed by inoculation of the bacterial colonies separately into 250 mL of trypticase soy broth in conical flask (500 mL). At each time interval 5mL of culture broth was centrifuged at 12000 rpm for 30 min. Then 100 µL of each supernatant was added into the each well in the cultivated Mueller Hinton agar by bioassay bacterium and incubated at 35°C. After 24h, the observation of growth inhibition zone considered bacteriocin activity.

Effect of temperature and pH on bioactive compounds*:* Activity of partial purified bioactive compounds at different temperatures was tested by overlaid of 10mL of aliquot bioactive compounds using paraffin oil. The tubes were incubated at 40, 50, 60 and 70°C for 30 min then, 100 µL of each bioactive compound was added into the well of cultivated Mueller Hinton agar by bioassay strain of *E. coli* (Sharma et al. 2009). After 24h, observation of zone of inhibition was considered bacteriocin activity. To evaluate the effect of different pHs on activity of the bacteriocins one millilitre of partial purified bacteriocins was adjusted to pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 by diluted NaOH and HCl for 1h and readjusted to pH 7.0. Then, 100 µL of each bacteriocin was added into the well of cultivated Mueller Hinton agar by bioassay strain of *E. coli* (Sharma et al. 2009). After 24h, observation of zone of inhibition was considered bacteriocin activity.

Effect of proteinase K on activity of the bioactive compounds: Bioactive compounds produced by the isolates were treated with proteinase K at a final concentration of 1 mg/mL for 2 h at 37°C. Then, 100 µL of each bacteriocin was added into the well on the Mueller Hinton agar cultivated by bioassay strain of *E. coli* . After 24h, observation of zone of inhibition was considered bioactive compounds activity (Hammami et al. 2009).

Determination of arbitrary units (AU) and molecular weight of bioactive compounds: In the present study Arbitrary Unit of bioactive compounds was determined in two steps before and after partial purification. Arbitrary Unit of bioactive compounds was determined by cultivation of the isolates in nutrient broth and incubated at 37°C for 24 h. The tubes were centrifuged for 15 min at 10,000 rpm then; the supernatants were double diluted 1/2, 1/4, 1/8, 1/16, 1/32, 1/64/1/128 and 1/256 using distilled water. After that, 100 µL of each dilution was added into the well in the cultivated Mueller Hinton agar by bioassay bacterium. The Arbitrary Unit was determined by the reciprocal of the highest dilution showing antimicrobial activity (Van Reenen et al. 1998).

Antimicrobial spectrum of bioactive compounds: Antimicrobial activity of bacteriocins against various pathogenic bacteria and fungi viz., *Bacillus cereus* PTCC 1137*, Pseudomonas aeruginosa* PTCC 1556, *Salmonella typhi* PTCC 1230, *Candida albicans* PTCC 5027 and *Aspergillus niger* PTCC 5011 was assessed by well diffusion agar method. To perform the test, each bioassay strain was full cultivated on Mueller Hinton agar and two wells were made in plate agar using sterile sharp borer. Then, 100 µL of purified bioactive compounds of BC10 was separately added into each well and the plate was incubated at 35°C. After 24h, clear zone of growth inhibition was measured and considered bioactive compounds activity. All experiments were carried out in three replicates.

RESULTS

Isolation and identification of bioactive compound producing bacteria: Out of 121 soil samples, two strains of *Brevibacillus* with potent activity for production of bioactive compound were isolated. Phenotypic and 16SrRNA sequencing identification of the isolates recognized them as *Brevibacillus reuszeri* (B12) and *Brevibacillus formosus* (B22) (Fig. 1).

Bioactive compounds of B12 and B22 showed antimicrobial property against *E. coli* with inhibition zone of 18 and 17 mm and *Staphylococcus aureus* with inhibition zone of 16 and 14 mm respectively.

Production of bioactive compound in different growth phases: The results obtained from production of bioactive compounds during growth phases of the isolates indicated production of antimicrobial compounds for both was started at 5th hour and reached to maximum level at 25th hour of bacterial growth. Hence, it may be interpreted that these compounds were produced in exponential phase of bacterial growth. In addition, our finding illustrated that antibacterial activity of the compounds moderately decreased after 25 hours of growth (Fig. 2).

Effects of enzyme, pH and temperature on activity of bioactive compounds: To determine the biochemical and biophysical properties of the bioactive compounds produced by the isolated strains, activity of the compounds was tested against proteinase K, different temperatures and pH values. The results obtained indicated that proteinase K inhibited antimicrobial activity of the compounds based on little or no growth inhibition zone of bioassay strain.

Although, full activity of the bioactive compounds retained at pH 7, potent activity of the bioactive compound produced by *Brevibacillus formosus* was at 35°C, and *Brevibacillus reuszeri* at 40°C.

Determination of arbitrary units of bioactive compounds: The results obtained from determination of AU before partial purification indicated that B12 and B22 were 64 and 32, however, after partial purification they were 128 and 64 respectively. This result shows that activity of both the bioactive compounds increased after partial purification.

Concerning to determination of molecular weight of the bioactive compounds it must be noted that the antibacterial property was related to the bands with 40 and 70 kDa for B12 and B22 respectively.

Antimicrobial spectrum of the bioactive compounds: The result obtained from antimicrobial spectrum of bioactive compounds B12 and B22 against some pathogenic microorganisms showed that *Aspergillus niger, Candida albicans* and *Pseudomonas aeruginosa* were resistant to both the bioactive compounds. However, *Salmonella typhi* and *Bacillus cereus* were sensitive to them. In addition, size of inhibition growth of all bacterial strains exhibited high activity of bioactive compound B12 as compared to B22 (Table 1).

DISCUSSION

The genus *Brevibacillus* has been introduced in 1996 (Shida et al. 1996). *Brevibacillus* is endospore forming bacteria and therefore could survive in the environment very well. On the other hand, the term probiotic was defined as "live

Table 1: Antimicrobial spectrum of bacteriocins against pathogenic microorganisms.

Bacteria	Inhibition zone diameter (mm) of bacteriocins	
	B12	B22
Bacillus cereus	17	14
Pseudomonas aeruginosa	- *	-
Salmonella typhi.	16	10
Candida albicans	-	-
Aspergillus sp.	-	-

*Resistant

ref|NR_040979.1| Brevibacillus formosus strain DSM 9885, 16S ribosomal RNA, artialsequence
Length=1486 Score = 516 bits (279), Expect = 1e-146, Identities = 302/313 (96%), Gaps = 3/313 (1%), Strand=Plus/Plus

```
Query 8      GGCGGGGTG-CT-ATACATGC-AGTCGAGCGAGTCTCTTCGGAGGCTAGCGGCGGACGGG 64
             ||||| ||| || |||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct 9      GGCGGCGTGCCTAATACATGCAAGTCGAGCGAGTCTCTTCGGAGGCTAGCGGCGGACGGG 68

Query 65     TGAGTAACACGTAGGCAACCTGCCTCTCAGACTGGGATAACATAGGGAAACTTATGCTAA 124
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 69     TGAGTAACACGTAGGCAACCTGCCTCTCAGACTGGGATAACATAGGGAAACTTATGCTAA 128

Query 125    TACCGGATAGGTTTTTGGATCGCATGATCCGAAAAGAAAAGGGGGCTTCGGCTGTCACTG 184
             |||||||||||||||||||||||||||| ||||||||||||| |||||||||||||||||
Sbjct 129    TACCGGATAGGTTTTTGGATCGCATGATNCGAAAAGAAAAGGCGGCTTCGGCTGTCACTG 188

Query 185    GGAGATGGCCTGCGGCGCATTGGCTAGTTGGTGGGGTAACGGCCTACCGAGGGGAGGAT 244
             ||||||||||||||||||||| |||||||||||||||||||||||||| ||| || |||
Sbjct 189    GGAGATGGCCTGCGGCGCATTAGCTAGTTGGTGGGGTAACGGCCTACCAAGGCGACGAT 248

Query 245    GCGTAGCCGACCTGAGAGGGTGACCGGCCACACTGGGACTGAGGCACGGCCCAGACTCCT 304
             ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct 249    GCGTAGCCGACCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT 308

Query 305    ACGGGAGGCAGCA 317
             |||||||||||||
Sbjct 309    ACGGGAGGCAGCA 321
```

ref|NR_029132.1| Brevibacillus reuszeri strain 39, 16S ribosomal RNA, partial sequence
Length=1419, Score = 542 bits (293), Expect = 2e-154, Identities = 300/304 (99%), Gaps = 1/304 (0%), Strand=Plus/Minus

```
Query 16     GTGTGGCCGGTCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGTAGGCCGTTACCC 75
             |||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 304    GTGT-GGCCGGTCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGTAGGCCGTTACCC 246

Query 76     CACCAACTAGCTAATGCGCCGCAGGCCCATCTCCCAGTGATAGCCGAAGCCATCTTTTCT 135
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 245    CACCAACTAGCTAATGCGCCGCAGGCCCATCTCCCAGTGATAGCCGAAGCCATCTTTTCT 186

Query 136    TTTCGGATCATGCGATCCAAAAACCTATCCGGTATTAGCATAAGTTTCCCTATGTTATCC 195
             ||||||| |||||| |||| ||||||||||||||||||||||||||||||||||||||||
Sbjct 185    TTTCGGACCATGCGNTCCANAAACCTATCCGGTATTAGCATAAGTTTCCCTATGTTATCC 126

Query 196    CAGTCTGAGAGGCAGGTTGCCTACGTGTTACTCACCCGTCCGCCGCTAGCCTCCGAAGAG 255
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 125    CAGTCTGAGAGGCAGGTTGCCTACGTGTTACTCACCCGTCCGCCGCTAGCCTCCGAAGAG 66

Query 256    ACTCGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCGTCCTGAGCCAGGATCAAA 315
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 65     ACTCGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCGTCCTGAGCCAGGATCAAA 6

Query 316    CTCT 319
             ||||
Sbjct 5      CTCT 2
```

Fig.1: Alignment analysis of 16srRNA..

microorganisms administered in adequate amounts which confer a health effect on the host" (FAO/WHO 2001). However, the published reports concerning to health effects of sporeforming bacteria in humans are less. But several commercial products containing sporeformer bacteria have been introduced for human consumption (Sanders et al. 2003). Antimicrobial compounds with pathogen-inhibiting effect exhibited by probiotic bacteria, and such compounds have been shown to be produced by some sporeformers. In this regard *Brevibacillus* can be considered as special sporeforming bacteria for introducing as a probiotic agent, however, information concerning to its ability for production

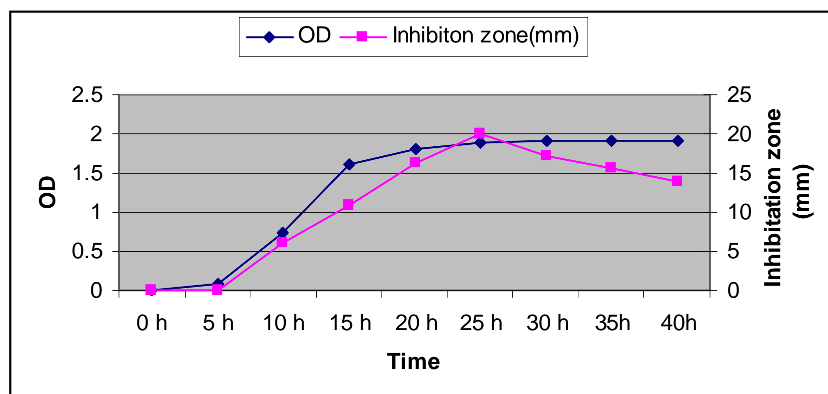

Fig. 2: Production of bioactive compounds during growth of *Brevibacillus formosus*. Each point represents the mean of three independent experiments.

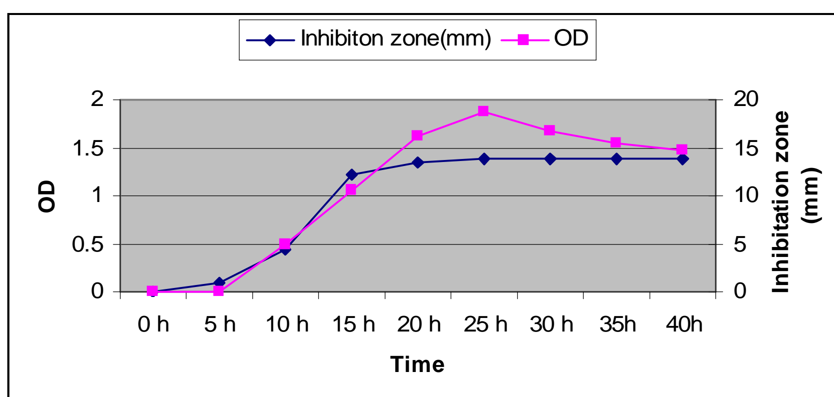

Fig. 3: Production of bioactive compounds during growth of *Brevibacillus reuszeri*. Each point represents the mean of three independent experiments.

of bioactive compounds is less (Lee et al. 2001). Therefore, the present study was conducted to evaluate capability of *Brevibacillus* for production of bioactive compounds. To achieve to this information in total, two strains of *Brevibacillus formosus* and *Brevibacillus reuszeri* with potent activity for production of bioactive compounds were isolated. These compounds exhibited antimicrobial activity against some Gram positive bacteria viz., *Bacillus cereus* and *Staphylococcua aureus* and Gram negative bacteria such as *E. coli* and *Salmonella typhi*. However, neither *Pseudomonas aeruginosa* nor fungi (*Candida albicans* and *Aspergillus niger*) were sensitive to both the bioactive compounds. In addition, characterization of the bioactive compounds indicated that activity of the compounds when treated with proteinase K was inhibited. Although, the best activity for both the bioactive compounds was observed at pH 7. The potent activity of bioactive compounds B12 and B22 was at 35°C and 40°C respectively. Hence, based on our information antimicrobial activity of the compounds could be depended on their protein structure. On the other hand these metabolites produced in exponential phase of bacterial growth, which is verified by association of these compounds with primary metabolites. However after 25 hours of growth, the antagonistic activity declined. Probably this reduction may be attributed with degradation of the bioactive compounds by specific or non-specific proteases (Parente & Ricciardi 1999).

Overall, bioactive compounds produced by different strains of *Brevibacillus* showed potent activity against some pathogenic microorganisms other than fungi. Therefore, this ability along with fast growth character may consider these bacteria as good probiotic agents especially for commercial industries.

REFERENCES

Baserisalehi, M. and Bahador, N. 2012. Isolation and molecular identification of *Pseudomonas* spp. from soil samples and their evaluation for bacteriocin production. Asian Pacific Journal of Tropical Biomedicine, pp. 1-5.

FAO/WHO 2001. Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. Available from: http://www.fao.org/es/ESN/food/foodandfood_probio_en.stm. Accessed 1 July 2003.

Edwards, S. and Seddon, B. 2001. Mode of antagonism of *Brevibacillus brevis* against *Botrytis cinerea* in vitro. J. Appl. Microbiol., 91: 652-659.

Garrity, G. M. (ed.) 2009. The Firmicutes. Bergey's Manual of Systematic Bacteriology. 3 (2nd ed). New York, Springer, pp.-1450. British Library No. GBA561951.

Gillor, O., Nigro, L.M. and Riley, M.A. 2005. Genetically engineered bacteriocins and their potential as the next generation of antimicrobials. Curr. Pharm. D., 11: 1067-1075.

Hammami, I., Rhouma, A., Jaouadi, B., Rebai, A., and Nesme, X. 2009. Optimization and biochemical characterization of a bacteriocin from a newly isolated *Bacillus subtilis* strain 14B for bio-control of *Agrobacterium* sp. strains. Lett. Appl. Microbiol., 48: 253-260.

Lee, K. H., Jun, K.D., Kim, W.S. and Paik, H.D. 2001. Partial characterization of polyfermenticin SCD, a newly identified bacteriocin of *Bacillus polyfermenticus*. Lett. Appl. Microbiol., 32: 146-151.

Oscariz, J.C. and Pisabarro, A.G. 2001. Classification and mode of action of membrane-active bacteriocins produced by Gram-positive bacteria.. International Microbiology, 4(1): 13-19.

Parente, E. and Ricciardi, A. 1999. Production, recovery and purification of bacteriocins from lactic acid bacteria. Appl. Microbiol. Biotechnol. 52: 628-638.

Sanders, M.E., Morelli, L. and Tompkins, T.A. 2003. Spore formers as human probiotics: *Bacillus, Sporolactobacillus* and *Brevibacillus.* Comprehensive Reviews in Food Safety Institute of Food Technologists, pp. 101-110.

Sharma, N., Kapoor, G., Gautam, N. and Neopaney, B. 2009. Characterization of a partially purified bacteriocin of *Bacillus* sp. MTCC 43 isolated from rhizosphere of radish (*Raphanus sativus*) & its application as a potential food biopreservative. Journal of Scientific & Industrial Research, 68: 881-886.

Shida, O., Takagi, H., Kadowaki, K. and Komagata, K. 1996. Proposal for two new genera, *Brevibacillus* gen.nov. and *Aneurinibacillus* gen. nov. Int. J. Syst. Bacteriol., 46: 939-946.

Van Reenen, C.A., Dicks, L.M.T. and Chikindas, M.L. 1998. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. Journal of Applied Microbiology, 84: 1131-1137.