Functional assessment of subtilosin A against *Aeromonas* spp. causing gastroenteritis and hemorrhagic septicaemia

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Anti-*Aeromonas* and cell membrane lytic bacteriocin substance, subtilosin A producing *Bacillus subtilis* VT03 was explored. Strain VT03 was isolated from freshwater fish (*Tilapia*) intestine and screened for its antimicrobial activity against four pathogenic strains of *Aeromonas* spp. causing gastroenteritis and hemorrhagic septicaemia. Isolate (VT03) was identified showing inhibition in agar spot assay. The strain VT03 was the one exhibiting strong inhibition and identified as *Bacillus subtilis* using 16S rRNA sequencing. Cell free supernatant (CFS) of the strain VT03 was active against pathogenic strains of *Aeromonas* spp, subsequently CFS was partially purified and designated as PPB-VT03 showing inhibition against *A. hydrophila* ATCC 49140. PPB-VT03 completely lost its activity upon treating with proteinase K revealing that the defense molecule could be proteinaceous in nature. Based on polymerase chain reaction (PCR), functional gene coding for subtilosin A (*sboA*) was found to be present whereas subtilin (*spaS*) was absent. The role of partially purified bacteriocin of isolate VT03 (PPB-VT03) through FTIR and SEM analysis revealed the activity of cell lysis. The study demonstrated the potential use of subtilosin A producing *Bacillus subtilis* as a potent source for antibacterial peptide.

Keywords: *Aeromonas* spp., *Bacillus subtilis*, subtilosin A, FTIR, SEM

Introduction

The need for integrated disease management is emphasized largely since commercial aquaculture is being affected by bacterial diseases. Pathogens that are envisaged in infecting aquatic products belong to the genera *Aeromonas*, *Nocardia*, *Streptococcus* and *Vibrio*. Even though bacterial pathogens are opportunistic, *Aeromonas hydrophila* is the challenging as well as the most harmful bacteria causing severe gill and skin diseases among freshwater fish species[1].

The deadly hemorrhagic septicemia and epizootic ulcerative syndrome are the common diseases due to *A. hydrophila* and exposing symptoms like local hemorrhages in the gills, anal area, dropsy, blisters, abscesses, scale protrusion, exophthalmia, tail rot, fin rot, abdominal swelling, anemia, accumulation of ascetic fluid, damage to the organs, notably kidney and liver[2]. Bacterial pathogen *A. hydrophila* could be the foremost contributing factor of ulcerative disease in cultured fish in Indo-Pacific region[3], Thailand and Malaysia[4].

Bacteriocins are ribosomally synthesized peptides which have major impacts on the growth and survival of other microorganisms. Colicins come under the category of bacteriocin[5], and most of the Gram-positive bacteria secrete antimicrobial peptides. Strains of *Bacillus* spp. are 'generally recognized as safe'(GRAS) bacteria[6] and produces lantibiotic bacteriocins including *Bacillus stearothermophilus*[7], *B. megaterium*[8] and *B. subtilis*[9]. Bacteriocins could be a possible alternative for xenobiotics or antibiotics and they could defend to control the pathogenic strains.

The present study was designed to investigate the role of bacteriocin substances of *Bacillus subtilis* VT03, possessing narrow spectrum against fish pathogenic strains. It was characterized using PCR detection conferring the presence of subtilosin A.

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The action against *A. hydrophila* was observed using scanning electron microscopy. The observation would make a pavement that subtilosin could act against fish pathogenic strain causing gastroenteritis and hemorrhagic septicaemia.

Materials and Methods

Bacterial Strains and Culture Conditions

Bacterial strain VT03[10] of normal microflora from the gut of freshwater fish tilapia (*Oreochromis mossambicus*) was isolated and screened for the presence of antagonistic activity against four strains of *Aeromonas* spp. VT03 exhibited antagonistic activity against *Aeromonas* spp. in agar spot assay. The 16S rRNA gene of the strain was submitted and assigned accession no. as KC512905 in the GenBank database. The promising strain VT03 was further studied and maintained in *Bacillus* agar. Pathogenic strains such as *A. hydrophila* (ATCC 49140), *A. hydrophila* (MTCC 1739), *A. sobria* (MTCC 3613) and *A. hydrophila* (OIE Reference laboratory, Vellore) were maintained in tryptic soy agar (TSA) (Himedia, India).

Antibiogram Test

In order to assess the antibiotic sensitivity of the strain VT03, the antibiotic discs (ampicillin, amoxycillin, cephalexin, gentamicin, penicillinG and streptomycin) were placed on Mueller Hinton Agar (MHA) plates seeded with 6 hours culture of VT03 and incubated for 24 h at 37°C[11]. The plates were observed for the zone of inhibition and the pattern was categorized based on the data rendered by the manufacturer.

Antibacterial Activity of the Cell Free Supernatant (CFS) (Well Diffusion assay)

Antimicrobial activity was assessed by standard disc and well diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS). An active culture of VT03 was screened for the ability of growth inhibition against four indicator strains mentioned above. An overnight culture of the strain VT03 was centrifuged to get cell-free supernatant (CFS) at $6,500 \times$ g. The pH of the CFS was adjusted to 7.0 using 1N NaOH. The antibacterial activity of this neutralized CFS was evaluated using agar well diffusion assay[12].

Production and Purification of Bacteriocin Substances from CFS-VT03

Strain VT03 was cultivated in nutrient broth for 18 h at 37°C and pelleted down at $6,500 \times$ g for 10 min. An equal volume of chloroform was added to the CFS

(CFS-VT03) under continuous stirring and followed by centrifugation to separate the chloroform extract. It was dehydrated and concentrated using lyophilizer (Lark Innovative Fine Teknowledge, India). The lyophilized content was re-suspended in sterile distilled water and designated as PPB-VT03 (partially purified bacteriocin – VT03)[13].

Antibacterial Activity of PPB-VT03

The antibacterial activity of the PPB-VT03 was tested using agar well diffusion assay[12] with *A. hydrophila* (ATCC 49140) as an indicator organism. PPB-VT03 was treated with proteolytic enzyme like proteinase K (P2308; Sigma) (in 0. 01 M phosphate buffer, pH 7. 0)[14]**.**

PCR Detection of Bacteriocin Genes from VT03

Genomic DNA was extracted from overnight cultures of VT03[15]. PCR reaction was performed to assess the presence of bacteriocin substances (subtilin and subtilosin) produced by the strain VT03. To specifically recognize the functional genes of subtilin *(spaS)* and subtilosin *(sboA)*, two of the primers were used[16] as follows:

Subtilin *spaS-f* (Forward)
(5'-TGTCATGGTTACAGCGGTATCGGTC-3')
spaS-r (Reverse)
(5'- AGTGCAAGGAGTCAGAGCAAGGTGA-3')
Subtilosin *sboA-f* (Forward)
(5'-CATCCTCGATCACAGACTTCACATG-3')
sboA-r (Reverse)
5'-CGCGCAAGTAGTCGATTTCTAACAC-3')

A reaction mixture of 50 μl containing 10 ng of genomic DNA, PCR mix (*Taq* buffer 10X, 25 mM $MgCl_2$, 25 mM dNTPs and *Taq* polymerase) (Ampliqon) and 20 μM of both forward and reverse primers (Eurofins Genomics) was prepared. A PCR temperature profile as 94°C for 1 min was used initially; then 30 cycles of 94°C for 1 min, 50°C for 30 sec, 72°C for 1 min and a final extension step at 72°C for 10 min was used. PCR products were sequenced and submitted to GenBank under the accession no. KP279466.

Fourier-Transform Infrared Sectroscopy (FTIR)

To study the mode of action of the bacteriocin substances PPB-VT03 on the cell membrane, the PPB-VT03 treated *Aeromonas hydrophila* (ATCC 49140) was subjected to FTIR analysis[14]. The cells were pelleted and treated with 1 ml of PPB-VT03 preparation of *Bacillus subtilis*. The treated and

untreated cells of the indicator organism were washed thrice with distilled water. The washed cells were lyophilized to remove moisture and powdered. The cells were mixed with finely grounded potassium bromide and the FTIR spectrum was recorded using a FTIR spectrometer (Perklin Elmer, USA).

Scanning Electron Microscopy (SEM)

To study the morphology of the indicator organism and to determine the mode of action of the PPB-VT03, SEM analysis was carried out[14]. To discover the effect of PPB-VT03 on cell morphology, the cell pellet of *A. hydrophila* (ATCC 49140) grown in tryptic soy broth for 12 h at 37°C was suspended in the PPB-VT03 preparation (1 ml) and incubated for 30 min. The PPB-VT03 treated and untreated cells were processed for SEM. The cells were harvested by centrifugation at 6,500 × g for 15 min and fixed using 2.5% (v/v) aqueous glutaraldehyde for 2 h. These cells were dehydrated using a gradient of ethyl alcohol (10 – 100%) and a final wash was done with absolute ethyl alcohol. The dried cells were gold plated and subjected to SEM (LEO 435-VP; Cambridge, UK).

Results

A. hydrophila VT03 strain possessing defense against the four pathogenic strains in agar spot assay was studied. CFS of VT03 strain was the most promising in well diffusion assay too. It was suspected whether extracellular protein present in the CFS of strain VT03 might be the reason and it was further studied. The strain VT03 was subjected to six antibiotics for their susceptibility pattern. The pattern of susceptibility to individual antibiotics was assessed. The strain VT03 was susceptible (S) to ampicillin, amoxycillin and gentamicin. In the case of cephalexin, penicillinG and streptomycin, the strain was moderately susceptible (MS).

After partial purification, the reproducible antibacterial spectrum of PPB-VT03 was assessed against an indicator strain, *A. hydrophila* (ATCC 49140). The partially purified bacteriocin substances possess the antagonism feature. It lost its activity completely upon proteinase K treatment. In specific, bacteriocin substance might be present corresponding to subtilin and subtilosin since the strain is *Bacillus subtilis*. It was suspected that one of these peptide molecules or both of the strain VT03 might be responsible for the inhibition activity against indicator strain.

The genes coding for subtilosin A *(sboA)* and subtilosin *(spaS)* have been cross checked in PCR detection. The results showed that the strain VT03 was found to be negative for the functional gene-encoding subtilin *(spaS)* and positive for subtilosin *(sboA)* functional gene (Fig. 1). FTIR spectra for wet dried (lyophilized) cell pellets of *A. hydrophila* (untreated) and *A. hydrophila* treated with bacteriocin substances PPB-VT03 is shown in Figure 2a & b. In PPB-VT03 treated cells, there were notable shifts in the absorbance depicting the changes occurring due to PPB-VT03 action on *A. hydrophila* cells.

The cells of *A. hydrophila* (untreated), showed region I to region IV as classical FTIR spectrum. The
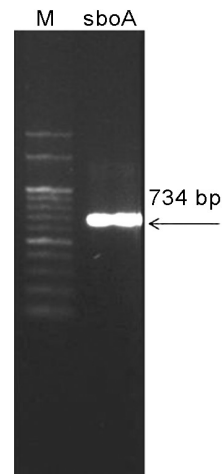

Fig. 1 — Confirmation of functional gene *sboA/spaS*. PCR testing shows the presence of the *sboA* functional gene along with 100 bp molecular marker.
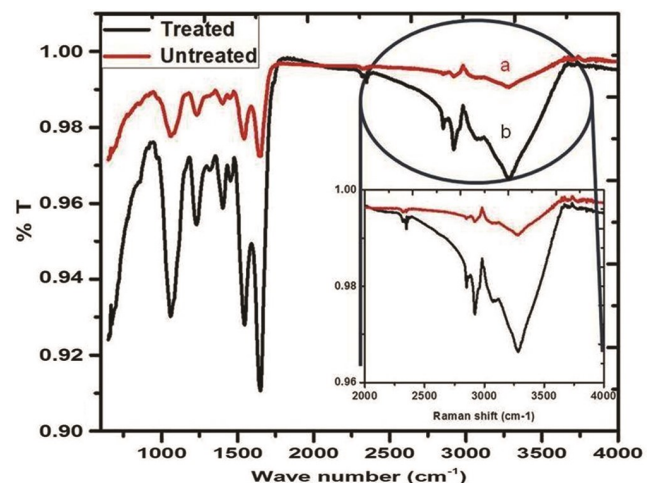

Fig. 2 — FTIR analysis of PPB-VT03 treated *Aeromonas hydrophila* ATCC 49140; a. (untreated) infrared spectra of untreated biomass (control); b. (treated) cell mass treated with bacteriocin like substances of *Bacillus subtilis*.
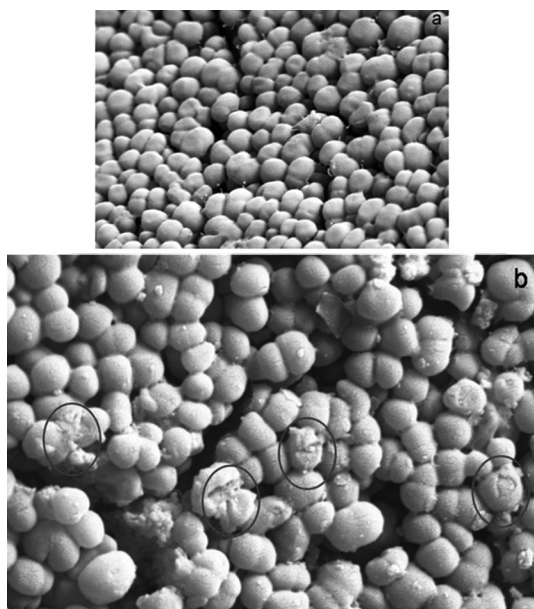
Fig. 3 — SEM of PPB-VT03 treated *A. hydrophila* ATCC 49140: a. Control; b. 30 min of treatment.

region I showed around 3000-2800 cm^{-1}, which represents cell membrane fatty acids. Region II shows amide I at 1650 cm^{-1} and amide II at 1550 cm^{-1}. Ultimate region IV shows around 1200 cm^{-1} absorption bands. The action of PPB was observed by SEM on *A. hydrophila* (ATCC 49140) cells upon 30 min of treatment with PPB-VT03 as shown in Figure 3. It is clearly understood that the ultimate mode of action of PPB-VT03 was the lysis of bacterial cell membrane.

Discussion

In the present study, *B. subtilis* (KC512905) have been isolated from the gut of freshwater fish Tilapia (*Oreochromis mossambicus)* which correlates the finding that bacilli have been isolated from carps[17]. The inhibitory action of CFS shown by VT03 in well diffusion assay was similar to the observations for *Alteromonas* sp.[18], *Bacillus* sp. BY-9[19] against the pathogenic strain *Vibriosis harveyi*. There were previous reports that non spore former, *Lactobacillus plantarum* 44a exhibited zone of inhibition against *A. hydrophila* and *Lactobacillus lactis* 18f showing inhibition zone of 11.2 ± 1.16 mm and 8.9 ± 2.1 mm against *A. hydrophila* and *Staphylococcus aureus* respectively[20]. The antagonism was acted against fish pathogenic strains by releasing substances with bactericidal or bacteriostatic effects. There are several factors which might be accountable for the inhibition of pathogenic strains like antibiotics, antimicrobial

peptides, bacteriocins, hydrogen peroxide, lysosymes, organic acids, proteases and siderophores[21].

The presence of subtilosin was further confirmed by PCR which is the best detecting tool for the rapid screening of class II bacteriocin producing strains[22]. PCR has also been employed to reveal the presence of the bacteriocins, nisin, pediocin and enterocin A in lactic acid bacteria isolated from traditional Thai fermented foods[23]. In PCR detection, *B. subtilis* strain is found to be having the gene corresponding to antibacterial peptide subtilosin A. Similar observation was made in the strain *Bacillus amyloliquefaciens*[24].

FT-IR spectra of bacterial cells can be used to analyze their total composition, including proteins, fatty acids, carbohydrates, nucleic acids, and lipopolysacharides. Earlier reports were made on bacterial systems having region I to IV[25]. Region I consisted of three noticeable peaks around 2960, 2925 and 2860 cm^{-1} [26-29]. In the case of region II, bands were observed corresponding to proteins and peptides[30]. Region III corresponds to fatty acids as well as proteins and phosphate-carrying molecules[26]. Region IV around 1200 cm^{-1} depicted the presence of bands typical of polysaccharides or carbohydrates of microbial cell walls[29-30].

It is noteworthy that the effect of bacteriocin substances PPB-VT03 on *A. hydrophila* showed all the IV regions in increased intensity (Fig. 2b). This could be concluded by the action of membrane leakage after bacteriocin treatment. Particularly, the C-H vibration regions in the treated cells are more pronounced, including the vast CH_2 and CH_3 bands at 2976 and 2876 cm^{-1}, respectively. Also, small shoulder peak at 3063 cm^{-1}, which is common characteristic feature for the CH mode in C = CH-R, might reflect an increased content of lipid residues containing unsaturated hydrocarbon and CH chains, as well as the bending CH (1381 cm^{-1}) and CH_2 scissoring mode (1457 cm^{-1})[31].

SEM has been widely employed in microbiological aspects to characterize the surface structure of biomaterials, to measure cell attachment and changes in morphology of bacteria[32]. Our results correlated with the findings[33] whereas the bacteriocins of *Pediococcus* were known to have bactericidal upon pore formation in the cytoplasmic membrane. The chronic exposure of antibiotics leads to cause resistance in microorganism which has let to have more interest towards bacteriocin substances. The peptides belonging to bacteriocin substances are

considered to be the primary defenders in food and clinical applications since the activity is often more-specific[34]. Our results would provide an insight towards a disease-free environment in aquatic industries.

Conclusion

Antimicrobial peptide is the need of the hour to replace antibiotics/xenobiotics to eradicate pathogenic strains. Bacteriocins are reported with its uniqueness of antimicrobial nature. In the present study, subtilosin A producing *B. subtilis* strain having narrow spectrum against opportunistic pathogenic strains *Aeromonas* spp. isolated from freshwater fish gut. Gene conferring subtilosin A was confirmed by gene specific primers. Ultimately, the action of partially purified bacteriocin was studied using FTIR and SEM. The present study revealed the cell lysis of *Aeromonas* spp. with the help of subtilosin A. Further studies are needed to understand the interaction between subtilosin A and extracellular proteins of *Aeromonas* spp. prior to lysis.

Acknowledgements

References

1   Smith P, Break points for disc diffusion susceptibility testing of bacteria associated with fish diseases, a review of current practice, *Aquacult*, 261 (2006) 1113–1121.

2   Rahman M H, Huys G, Rahman M, John A M, Kuhn I *et al*, Persistence transmission and virulence characteristics of *Aeromonas* strains in a duckweed aquaculture-based hospital sewage water recycling plant in Bangladesh, *Appl Environ Microbiol*, 3 (2007) 1444–1451.

3   Tonguthai K, A preliminary account of ulcerative fish diseases in the Indo-Pacific Region (a comprehensive study based on Thai experiences). National Inland Fisheries Institute, Bangkok, (1985) 39.

4   Torres J L, Shariff M & Law A T, Identification and virulence screening of *Aeromonas* sp. isolated from healthy and epizootic ulcerative syndrome infected fish. In: Proceedings of the Society of Asian Fisheries Forum, Tokyo, Japan, (1989) 17-22.

5   Pugsley A P, The ins and outs of colicins, *Microbiol Sci*, 1 (1984) 168-175.

6   Sharp R J, Scawen M D & Atkinson T, Fermentation and downstream processing of *Bacillus*. In: *Bacillus* Harwood, C. R. (Eds), New York. Plenum Press, (1989) 255-292.

7   Sharp R J, Bingham A H A, Comer M J & Atkinson A, Partial characterization of a bacteriocin (thermocin) from *Bacillus stearothermophilus* RS93, *J Gen Microbiol*, 111 (1979) 449-451.

8   Kiss A, Baliko G, Csorba A, Chuluunbaatar T, Medzihradszky K F *et al*, Cloning and characterization of the DNA region responsible for megacin A-216 production in *Bacillus megaterium*, 216, *J Bacteriol*, 190 (2008) 6448-6457.

9   Hansen M E, Wangari R, Hansen E B, Mijakovic I & Jensen P R, Purification and partial amino acid sequence of thuricin S, a new Anti-Listeria bacteriocin from *Bacillus thuringiensis*, *Can J Microbiol*, 53 (2007) 284-290.

10   Vignesh V, Sathiyanarayanan G, Sathishkumar G, Parthiban K, Sathish Kumar K *et al*, Formulation of iron oxide particles using exopolysaccharide: Evaluation of their antibacterial and anticancer activity, *RSC Adv*, 5 (2015) 27794.

11   Ghrairi T, Frere J, Berjeaud J M & Manai M, Purification and characterization of bacteriocins produced by *Enterococcus faecium* from Tunisian rigouta cheese, *Food Control*, 19 (2008) 162–169.

12   James G, Cappuccino, Natalie Sherman, 2009. Microbiology - A Laboratory Manual, 7th Edition.

13   Geis A, Singh J & Teuber M, Potential of lactic Streptococci to produce bacteriocin, *Appl Environ Microbiol*, 45 (1983) 205-211.

14   Halami P M, Badarinath V, Sundru Manjulata Devi & Vijayendra S V N, Partial characterization of heat stable, Anti-Listerial and cell lytic bacteriocin of *Pediococcus pentosaceus* CFR SIII isolated from a vegetable source, *Ann Microbiol*, 61 (2010) 323–330.

15   Kannan M, Suganya T, Arunprasanna V & Krishnan M, An efficient method for extraction of genomic DNA from insect gut bacteria-culture dependent, *Curr Res Microbiol Biotech*, 3 (2015) 550-556.

16   Velho R V, Basso A P, Segalin J, Costa-Medina L F & Brandelli A, The presence of *sboA* and *spaS* genes and antimicrobial peptides subtilosin A and subtilin among *Bacillus* strains of the Amazon basin, *Genet Mol Biol*, 36 (2013) 101-104.

17   Sen R & Srinivasa Babu K, Modeling and optimization of the process conditions for biomass production and sporulation of a probiotic culture, *Process Biochem*, 40 (2005) 2531-2538.

18   Kumar R, Mukherjee S C, Prasad K P & Pal A K, Evaluation of *Bacillus subtilis* as a probiotic to Indian major carp *Labeo rohita* (Ham), *Aquac Res*, 37 (2006) 1215-1221.

19   Abraham T J, Antibacterial marine bacterium deters luminous vibriosis in shrimp larvae. *Naga, Worldfish Center Quarterly*, 27 (2004) 3-4.

20   Rengpipat S, Phianphak W, Piyatiratitivorakul S Menasaveta P, Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth, *Aquacult*, 167 (1998) 301-313.

21   Galindo A B, *Lactobacillus plantarum* 44A as a live feed supplement for freshwater fish. Ph.D. diss, Wageningen Univ., Wageningen, Netherlands (www.gcw. nl/dissertations/content. cfm), (2004).

22   Babasaki K, Takao T, Shimonishi Y, Kurahashi K & Subtilosin A, a new antibiotic peptide produced by *Bacillus subtilis* 168: Isolation, structural analysis and biogenesis, *J Biochem*, 98 (1985) 585-603.

23   Yi H, Zhang L, Tuo Y, Han X & Du M, A novel method for rapid detection of class IIa bacteriocin-producing lactic acid bacteria, *Food Control*, 21 (2010) 426-430.

24 Suwanjinda D, Eames C & Panbangred W, Screening of lactic acid bacteria for bacteriocins by microbiological and PCR methods, *Biochem Mol Biol Educ*, 35 (2007) 364-369.

25 Sutyak K E, Wirawan R E, Aroutcheva A A & Chikindas M L, Isolation of the *Bacillus subtilis* antimicrobial peptide subtilosin from the dairy product-derived *Bacillus amyloliquefaciens*, *J Appl Microbiol*, 104 (2008) 1067–1074.

26 Jiang W, Saxena A, Song B, Ward B B, Beveridge T J *et al*, Elucidation of functional groups on Gram-positive and Gram-negative bacterial surfaces using infrared spectroscopy, *Langmuir*, 20 (2004) 11433-11442.

27 Lu X, Liu Q, Wu D, Al-Qadiri, H M, Al-Alami N I *et al*, Using of infrared spectroscopy to study the survival and injury of Escherichia coli O157 : H7, Campylobacter jejuni and Pseudomonas aeruginosa under cold stress in low nutrient media, *Food Microbiol*, 28 (2011) 537–546.

28 Lu X, Rasco B A, Jabal J M F, Aston D E, Lin M *et al*, Investigating antibacterial effects of garlic (Allium sativum) concentrate and garlic-derived organosulfur compounds on *Campylobacter jejuni* by using fourier transform infrared spectroscopy, raman spectroscopy and electron microscopy, *Appl Environ Microbiol*, 77 (2011) 5257–5269.

29 Alverez-Ordóñez A, Halisch J & Prieto M, Changes in Fourier transform infrared spectra of Salmonella enterica serovars typhimurium and Enteritidis after adaption to stressful growth conditions, *Int J Food Microbiol*, 142 (2010) 97–105.

30 Zoumpopoulou G, Papadimitriou K, Polissiou M G & Tarantilis P A, Detection of changes in the cellular composition of *Salmonella enterica* serovar typhimurium in the presence of antimicrobial compound (s) of *Lactobacillus* strains using fourier transform infrared spectroscopy, *Int J Food Microbiol*, 144 (2010) 202–207.

31 Davis Rm & Mauer L J Fourier transform infrared (FT-IR) spectroscopy : A rapid tool for detection and analysis of foodborne pathogenic bacteria. In Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology Volume 2. Edited by Méndez-Vilaz A. Badajoz, Spain: Formatex, (2010) 1582–1593.

32 Kamnev A A, Antonyuk L P, Matora L Y, Serebrennikova O B, Sumaroka M V *et al*, Spectroscopic characterization of cell membranes and their constituents of the plant associated soil bacterium Azospirillum brasilense, *J Mol Struct*, 480–481 (1999) 387–393.

33 Tekehiko Kenzaka & Katsuji Tani, (2012), Scanning electron microscopy imaging of bacteria based on nucleic acid sequences, scanning electron microscopy, Dr. Viacheslav Kazmiruk (Ed), InTech, ISBN: 978-953-51-0092-8,http://www.intechopen.com/books/scanning-electron microscopy/scanning-electronmicroscope-imaging-of-bacteria-based-on-nucleic-acid-sequences.

34 Bhunia A K, Johnson M C, Ray B & Kalchayanand N, Mode of action of pediocin AcH from *Pediococcus acidilactici* H on sensitive bacterial strains, *J Appl Bacteriol*, 70 (1991) 25-33.