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Bacteriocin Producing *Bacillus thuringiensis* and its Effect on Human Pathogens

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Sixty five *Bacillus thuringiensis* strains were isolated from soils of the western ghats of Maharashtra. From sixty five isolates of *B. thuringiensis*; ten *B. thuringiensis* strains were screened for their cry gene profile (cry2, cry3 and cry6) and capacity to express bacteriocin-like agents. *B. thuringiensis* isolate no.9, showed antagonistic activity towards *Corynebacterium diphtheriae, Rothia dentocariosa, Moraxella catarrhalis, Neisseria polysaccharea, Staphylococcus aureus, Streptococcus pyogenes,* and other *Bacillus* sp.; The partially purified bacteriocin (PPB) obtained from isolate no.9 by salt precipitation was studied by SDS-PAGE had an apparent Molecular weight of 43kDa. Effect of temperature and pH on activity of PPB was studied. This novel identified and characterized partially purified bacteriocin can be effective in control of human pathogen such as *Coryne. diphtheriae* it may play an interesting role in therapeutic science.

Key words: *Bacillus thuringiensis,* Bacteriocin, SDS PAGE, *Corynebacterium diphtheriae.*

Bacteriocins are inhibitory peptides or proteins, produced by different groups of bacteria, which have bactericidal effects on micro-organisms closely related to the producer. Bacteriocins especially those produced by lactic acid bacteria (LAB) are of interest, because of their potential use as food additives and their efficiency for the biological control of spoilage and pathogenic organisms[1].

These substance produced by organisms selectively interferes with the growth of other organisms and are categorized as bioactive molecules. The bacteriocins comprises the ribosomally synthesized proteinaceous compounds released extracellularly by bacteria that can be shown to interfere with the growth of other bacteria, typically including some that are closely related to the producing bacterium and to which the producer cell expresses a degree of specific immunity. The genus *Bacillus* encompasses a number of bacteriocinogenic species, such as *B. subtilis* which produces subtilin[2] and subtilosin[3], *B. coagulans* which produces coagulin[4], *B. megaterium* which produces megacin[5] and *B. thermoleovorans* which produces thermoleovorin [6].

B. thuringiensis is a Gram positive, soil dwelling bacterium of genus *Bacillus*. *B. thuringiensis* is widely used in agriculture for the control of many insect parasites. It is characterized by the production of crystal proteins (δ-endotoxins) with a specific activity against certain insect species[7], nematodes, mites and protozoa8. Moreover, a number of extracellular

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compounds are produced by *B. thuringiensis*, such as phospholipases, chitinases, proteases[9], β- exotoxins, vegetative insecticidal proteins and antibiotic compounds with antifungal activity 10.

Bacteriocins associated with *B. thuringiensis*, and the information concerning the chemical nature, activity spectrum and characteristics of these bacteriocins is not studied extensively. Few bacteriocins have been partially characterized from *B. thuringiensis*: thuricin (950 kDa) from strain HD2 [11], tochicin (10.5 kDa) from strain HD868 [12], thuricin 7 (11.6 kDa) from strain BMG1.7 [13], thuricin 439A and thuricin 439B (2.9 and 2.8 kDa, respectively) from strain B439 [14]; Entomocin 9 (12.4) kDa from *B. thuringiensis ssp. entomocidus HD9.*

In the present study a novel bacteriocin (PPB) produced by *B. thuringiensis* strain ME-9, with a broad-spectrum activity against various human pathogens such as *Coryne. diphtheriae, Rothia dentocariosa, Mor. catarrhalis, N. polysaccharea, Staph. aureus, Strep. pyogenes,* and other *Bacillus* sp.; was detected.

MATERIAL AND METHODS

Isolation and identification of *Bacillus thuringiensis* from soil

Sixty five isolates of *B. thuringiensis* were isolated from soils of the western ghats of Maharashtra *viz.* Vasai, Bhiwandi region. The isolation of *B. thuringiensis* was done using Traver's method[15]. In this method germination of *B. thuringiensis* spores was selectively inhibited by sodium acetate, while most of the undesired sporeformers germinated. The heat resistant spores of *B. thuringiensis* were selected by heating it at 80°C for 3 mts, while all the nonsporulated were eliminated. Further selective medium i.e. Modified Glucose Medium[16], and Nutrient agar Penicillin medium were used for the isolation of *B. thuringiensis*.

Identification of *Bacillus thuringiensis* strains

The identification of the isolates was carried out by the following biochemical tests.

Biochemical tests: starch hydrolysis; urease production; sucrose fermentation; esculin utilization; lecithinase production; casein hydrolysis and gelatinase test to identify *B. thuringiensis* for screening.

DNA isolation

The genomic DNA was extracted by Phenol: Chloroform method 17.Briefly, Cells were grown in Sterile Brain Heart Infusion broth, and were harvested by centrifugation at 10000 r.p.m. at 4°C for 10 minutes. Pellet was washed with buffer (10 mM l^{-1}Tris-HCl, pH7.8, 5 mM l^{-1} EDTA) and treated with Lysozyme (50 g l^{-1}). SDS Lysis solution (20% SDS in 50 mM l^{-1} Tris-HCl, pH 7. 8, 20 mM l^{-1} EDTA) was added to the lysed cells along with proteinase k (20g l^{-1}). Equal volume of Phenol: Chloroform was added to cell lysate and DNA was precipitated out by adding double volume of ice cold ethanol to the aqueous layer. The DNA content was calculated by measuring the absorbance at 260 nm and its quality was assessed by the 260:280 nm absorbance ratio and by electrophoresis on agarose gel.

PCR analysis

The presence of *cry* gene i.e. *cry*2; *cry*3; *cry*6 encoding for crystal proteins possessing insecticidal (Dipteran, Lepidopteran, Coleopteran) and nematocidal activity was determined using PCR technique18. The analysis was performed in a final reaction mixture of 12µl containing: 1.5µl of Taq assay buffer (10 X), 1.5µl of dNTP's (1mM), 3µl of Forward and Reverse Primers of concentrtion 5 picomoles each, specific for *cry* 2, *cry* 3, *cry* 6 (Table 1), 1.5µl $MgCl_2$, 0.375µl of Taq DNA Polymerase (obtained from Applied Biosystems Pvt. Ltd.), 1µl of Template DNA and 3.125µl of sterile distilled water. (Taq polymerase and dNTP's were obtained from Applied Biosystems and primers were obtained from Banglore Genei), Thermocycler (Applied Biosystems Gene Amp®PCR systems 2700) .The amplification system is given in Table 2.

Determination of Bacteriocin activity

Ten isolates of *B. thuringiensis* were screened for their ability to produce bacteriocin by agar spot method. The isolates were spotted on the nutrient agar plates which were initially swabbed with pathogenic culture and the same were incubated at 37°C for 24hrs. The zone of clearance around the isolate demonstrated the bacteriocin activity.

To rule out the cause of clearance or lysis because of lytic phages, reverse-side plate technique was applied. This technique is based on the fact that diffusion of bacteriocins is tridimensional. The producer strain was spotted

on the surface of nutrient agar. After suitable incubation, the agar was detached from the edges of the Petri dish with a sterile spatula. The plate was then inverted, and the petri dish was tapped sharply on the bench so that the agar disc falls into the lid. The sterile surface was uppermost in the lid, and the indicator strain was swabbed on this surface. After incubation, the zone of inhibition indicates bacteriocin activity[19].

Partial Purification of Bacteriocin

The isolate producing bacteriocin was inoculated in the nutrient broth and incubated in shaker condition at room temperature for 48 hrs. The cells were removed by centrifugation at 10000 r.p.m. at 4°C for 10 minutes and the supernatant was precipitated with ammonium sulphate at 50% saturation. The protein precipitate was obtained by centrifugation at 11,000 g for 30 min. The precipitate was dissolved (1/100 of the original volume) in 50 mM l^{-1} 1:1 sodium phosphate-buffered saline (PBS) pH 6.8 and intensively dialysed four times against PBS (pH 6.8) for 24 hrs in Spectra-Por no. 3 dialysis tubing. The Partially Purified Bacteriocin (PPB) was further tested for the antimicrobial activity by disc diffusion method.

Effect of pH and temperature on the antimicrobial activity of Partially Purified Bacteriocin (PPB) by Agar Cup method

To study the effect of temperature on inhibitory activity of partially purified bacteriocin, 500 microlitres of PPB were incubated at varied temperatures (4°C, RT, 37°C, 55°C) for 30 min. The pH stability was studied after storage of PPB for one day at 4°C in the following buffers: 10mM l^{-1} citrate buffer pH-3, 10 mM l^{-1} phosphate buffer pH-7, 10 mM l^{-1} Tris-HCl buffer pH-9. The test organism selected for the study was *Coryne. diphtheriae*.

SDS-PAGE analysis

The partially purified bacteriocin and standard molecular weight marker (Bangalore genei, range 29 – 205 kDa) were subjected to 7% SDS-PAGE. The procedure was performed by standard protocols 20 using Tris-glycine buffer. Following electrophoresis, gel was stained with coomassive blue R-250.

HPTLC analysis of Partially Purified Bacteriocin

Amino acid content was analyzed using HPTLC technique. Acid hydrolyzed PPB and the standard amino acids Leucine, Valine, Alanine, Lysine, Arginine, and Histidine were subjected to HPTLC analysis. For the analysis Partially purified bacteriocin, the standard amino acids 5mg (standard amino acids: Leucine, Alanine, Valine, Lysine, Arginine, Histidine obtained from Himedia, Mumbai, India) was dissolved in 10ml Methanol water mixture (1:1)},TLC Silica Gel Plate 554, Linomat 5 (sample applicator) solvent system, ninhydrin reagent, CAMAG TLC Scanner 3 were used.

RESULTS

Out of 120 *Bacillus* strains isolated from soil of western ghat region in Maharashtra, 65 isolates were identified as *B. thuringiensis* by biochemical tests, and its ability to produce the ä-endotoxin inclusion bodies. From the 65 isolates, 10 isolates were selected randomly and was subjected to PCR analysis to detect the presence of *cry*2, *cry*3, *cry*6 gene (Table 4) and screened for their ability to produce bacteriocin-like agents by agar spot method 20. Results of the agar spot method are given in Table 3 and the reference strains for respective cry genes used in PCR analysis are listed in Table 5. Out of the 10 isolates,

Table 1. *Cry* primers details

S. No.	Gene	Sequences	Size (bp)	References
1	*cry* 2	FP: GTTATTCTTAATGCAGATGAATGGGRP: CGGATAAAATAATCTGGGAAATAGT	689-701	Ben dov *et.al.*, 1997
2	*cry* 3	FP: AAACHGAAYTAACAAGAGACRP: AASTKAGWKGTWGAAGCATA	858	Masson *et.al.,* 1998
3	*cry* 6	FP: TAYGGTTTTAAAKKTGCTGGRP: TRAATYCTATTRAACAATCCTA	587	Masson *et.al.,* 1998

Table 2. PCR amplification conditions

Step	Temperature (°C)	Duration (min)	No. of cycles
Initial denaturation	94	5	1
Denaturation	94	1	
Annealng (For individual pair of primers)			
*Cry*2	51.6	1	39
*Cry*3	48	1	39
*Cry*6	45.4	1	39
Initial Extension	72	2	
Final Extension	72	20	1
Hold	4		

Table 3. Screening of isolates for the production of bacteriocin by agar spot method

S. No	Pathogens	Isolate 2	Isolate 3	Isolate 4	Isolate 6	Isolate 8	Isolate 9	Isolate 10
1	*Corynebacterium diphtheriae*	+	-	+	+	+	+	-
2.	*Rothia dentocariosa*	+	+	-	-	-	+	-
3.	*Moraxella catarrhalis*	-	+	-	-	+	+	-
4.	*Neisseria polysaccharea*	+	+	-	+	+	+	-
5.	*Staphylococcus aureus*	+	-	+	+	+	+	-
6.	*Streptococcus pyogenes*	-	-	-	-	-	-	-
7.	*Salmonella typhi*	+	-	-	-	-	-	-
8.	*Salmonella- paratyphi A*	-	-	-	-	-	-	-
9.	*Esherichia coli*	-	-	-	-	-	-	-
10.	*Proteus- vulgaris*	-	-	-	-	-	-	-
11.	*Proteus mirabilis*	-	-	-	-	-	-	-
12.	*Bacillus* species	+	-	-	+	+	+	+

Key:- : No Zone of Inhibition. + : Zone of Inhibition
Effect of bacteriocin from each isolate on human pathogens was checked by agar spot method. +/- sign shows inhibitory / non inhibitory effect of bacteriocin.

Table 4. Cry gene profile of isolates

S. No	cry gene	Isolate 2	Isolate 3	Isolate 4	Isolate 6	Isolate 8	Isolate 9	Isolate 10	Reference strain
1	*cry2*	-	-	-	-	-	-	-	+
2.	*cry3*	-	-	-	-	-	-	-	+
3.	*cry 6*	-	-	-	-	+	+	-	+

Key: +: gene present - : gene absent
Presence or absence of *cry* type of genes was studied by PCR based *cry* profile method. Isolate no. 8 and 9 showed presence of *cry6* gene.

Table 5. Reference strains used

S.No	cry gene	Reference strain
1	*cry2*	*Bacillus thurigiensis sub sp. krustaki (4D4)*
2.	*cry3*	*Bacillus thurigiensis sub sp. aizawai (HD 133)*
3.	*cry 6*	*Bacillus thurigiensis sub sp. krustaki (HD1)*

four isolates possessed bacteriocin like activity against *Coryne. diphtheriae, Rothia dentocariosa, Mor. catarrhalis, N. polysaccharea, Staph. aureus, Bacillus* sp., *Salm. typhi,* and *Strep. pyogenes.* Further from the four isolates, two isolates (isolate no.8 and isolate no. 9) were detected, having nematocidal activity encoded by *cry*6 gene [detected by PCR method] [21]. From these two isolates, Isolate no. 9 was selected because of its antimicrobial activity against *Coryne. diphtheriae, Rothia dentocariosa, Mor. catarrhalis, N. polysaccharea, Staph. aureus* and *Bacillus* spp.

PPB was partially purified from 48hr. old culture by ammonium sulphate precipitation followed by subsequent dialysis. The bacteriocin containing preparation i.e. PPB was examined for its sensitivity to pH variation (3-9) and heat (4-55°C).The Partially Purified Bacteriocin (PPB) was stable at a wide range of temperature (4-55°C) and pH (3-9) and maximum stability was observed at 55°C and pH 7. The identified bacteriocin is stable at pH 3; hence its activity against gastrointestinal tract infection can be of significance and can be used for the further study.

The molecular weight of Partially Purified Bacteriocin (PPB) was found to be 43kDa, approximately. The band had an apparent molecular

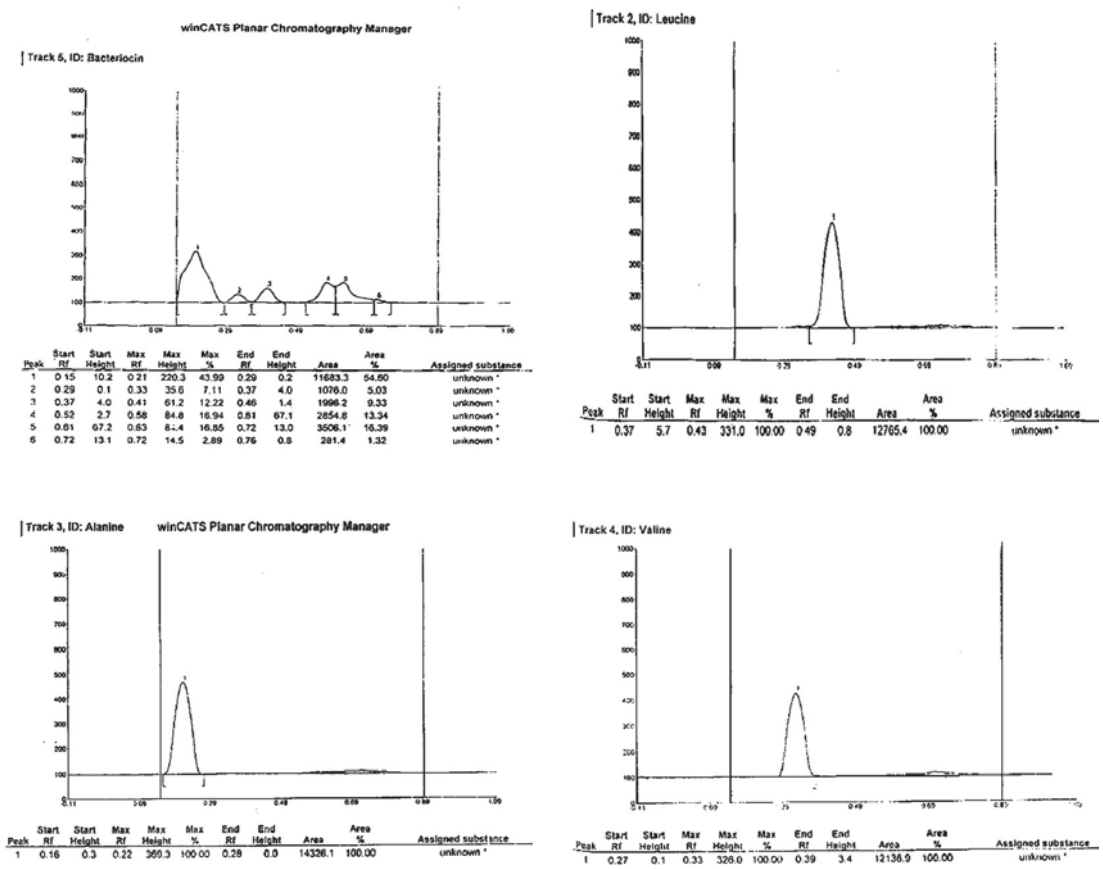

Fig 1. HPTLC of partially purified bacteriocin: Graph representing peaks of different substances in bacteriocin and their Rf values (Relative migration) is shown in Fig4 (A). Rf values of bacteriocin are compared with Rf values of standard amino acids like Leucine, Alanine, Valine, Lysine, Arginine, and Histidine. Rf value of peak 1 of bacteriocin showed highest similarity with the Rf value of standard amino acid Alanine (B). Whereas Rf values of peak 2 and peak 3 of bacteriocin showed highest similarity with the Rf values of standard amino acids Valine and Leucine respectively

mass of about 43kDa, as estimated by calculating the different *rf* (relative migration) value of standard proteins.

Amino acid content analysis (Fig.1), showed that bacteriocin contains amino acid Leucine and Valine in concentration 0.0035% and 0.001917%, respectively.

DISCUSSION

Out of the 65 isolates, four isolates possessed bacteriocin like activity against *Coryne. diphtheriae, Rothia dentocariosa, Mor. catarrhalis, N. polysaccharea, Staph. aureus, Bacillus* sp., *Salm. typhi,* and *Strep. pyogenes.* Further, two isolates (isolate no.8 and isolate no. 9) were detected; having nematocidal activity encoded by *cry*6 gene [detected by PCR method] 21. Isolate no. 9 was selected because of its antimicrobial activity against *Coryne. diphtheriae, Rothia dentocariosa, Mor. catarrhalis, N. polysaccharea, Staph. aureus* and *Bacillus* spp.

Bacteriocin was partially recovered from isolate no.9. The molecular weight of Partially Purified Bacteriocin (PPB) from isolate no.9 was found to be 43kDa, approximately. The result shows that the newly identified bacteriocin is different from thurcin, 950 kDa; thurcin 7, 11.6kDa; thuricin 439A, 2.9 kDa; and thuricin 439B, 2.8 kDa; tochicin, 10.5 kDa; Entomocin 9, 12.4 kDa 11, 12, 13, 14.

Amino acid analysis showed the presence of Leucine and Valine. These amino acids are polar nature therefore may be contributing to the hydrophilic nature of bacteriocin. Thus it can be concluded that, a novel bacteriocin produced by *B. thuringiensis* isolate no.9 is found to be relatively stable at varied temperature and pH. The antimicrobial activity of the newly identified bacteriocin against wide range of pathogens indicates its potential use in therapeutics.

REFERENCES

1. Delves-Broughton J., *Food Technol.,* **44**: 100-117 (1990).
2. Jansen E. F. and Hirschmann D. J., *Arch. Biochem*., **4**: 297-304 (1944).
3. Zheng G. and Slavik M. F**.,** *Lett. Appl. Microbiol*., **28**: 363-365 (1999).
4. Hyronimus B., Le Marrec C. and Urdaci M. C., *J. Appl. Microbiol.,* **85**: 42-50 (1998).
5. Von Tersch M. A. and Carlton B. C., *J. Bacteriol*., **155**(2): 866–871 (1983).
6. Novotny J. F. Jr. and Perry J. *J. Appl Environ Microbiol*., **58**(8): 2393-2396 (1992).
7. Beegle C. C. and Yamamoto T., *Can. Entomol.,* **124**: 587-616 (1992).
8. Feitelson J. S., Payne J. and Kim L., *Bio/Technol.* **10**: 271-275 (1992).
9. Lo¨vgren A., Shang M. - Y., Engstro¨m A., Dalhammmar G. and Lande´n R., *Mol. Microbiol.* **4**: 2137-2146 (1990).
10. Stabb E. V., Jacobson L. M. and Handelsman J. O., *Appl. Environ. Microbiol*., **60**: 4404-4412 (1994).
11. Favret M. E. and Yousten A. A., *J. Invertebr. Pathol*., **53**: 206-216 (1989).
12. Paik H. D., Bae S. S., Park S. H. and Pan J. G., *J. Ind. Microbiol. Biot*., **19**: 294-298 (1997).
13. Cherif A., Ouzari H., Daffonchio D., Cherif H., Ben Slama K., Hassen A., Jaoua S. and Boudabous A., *Lett. Appl. Microbiol*., **32**: 243-247 (2001).
14. Ahern M., Verschueren S. and Van Sinderen D., *FEMS, Microbiol. Letters*, **220**(1): 127-131 (2003).
15. Travers R. S., Martin P. A. W. and Reichelderfer C. F., *Appl. Environ. Microbiol*., **53**(6): 1263-1266 (1987)
16. Aronson A. I., Angelo N. and Holt S. C., *J. Bacteriol*., **106**: 1016-1025 (1971).
17. Sambrook J. and Russel, D. W., Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, New York, 6.4 - 6.12, (2001).
18. Sambrook J. and Russel, D. W., Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, New York, 8.4 - 8.24, (2001).
19. Parrot M., Caufield P. W. and Lavoie M. C., *Can. J. Microbiol*., **36**: 123-130 (1990).
20. Laemmli U. K., *Nature*, **227**: 680-685 (1970).
21. Bravo A., Sarabia S., Lopez L., Ontiveros H., Abarca C., Ortiz A., Ortiz M., Lina L., Villalobos F. J., Pena G., Valdez M., Soberon M. and Quintero R., *Appl. Environ. Microbiol.,* **64**(12): 4965-4972 (1998).
22. Tagg J. R., Dajani A. S., Wannamaker L. W. and Gray E. D., *J. Exp. Med*., **138**(5): 1168-1183 (1973).
23. Akiba T., Abe Y., Kitada S., Kusaka Y., Ito A., Ichimatsu T. and Katayama H., *Acta Cryst*., 2355-2357 (2004).
24. Apoyolo C. T., Drif L., Vassal J. M., Debarjac H., Bossy J. P., Leclant F. and Frutos R., *Appl.*

Environ. Microbiol., **61**(12): 4343-4347 (1995).
25. Bizani D. and Brandell A., *J. Appl. Microbiol*., **93**: 512-519 (2002).
26. Brousseau R., Saint-Onge A., Prefontaine G., Masson L. and Cabana J., *Appl. Environ. Microbiol*., **59**(1): 114-119 (1993).
27. Carrozzi N. B., Kramer V. C., Warren G. W., and Koziel M. G., U.S. patent, **5**: 204, 100. (1993)
28. Cherif A., Chehimi S., Limem F., Hansen B. M., Hendriksen N. B., Daffonchio D. and Boudabous A. *J. Appl. Microbiol*., **95**: 990-1000 (2003).
29. Eijsink V. G. H., Skeike M., Middelhoven P. H., Brurberg M. B. and Nes I. F., *Appl. Environ. Microbiol*., **64**(9): 3275-3281 (1998).
30. Gratia J. P., *Genetics*, **156**: 471-476 (2000).
31. Heng N. K. C., Wescombe P. A., Burton J. P., Jack R. W. and Tagg J. R., Bacteriocins: Ecology and Evolution, 45-92 (2007).
32. Jack R. W., Tagg J. R. and Ray B., *Microbiol. Rev.,* **59**(2): 171-200 (1995).
33. Kekessy D. A. and Piguet J. D., *Appl. Microbiol.,* **20**(2): 282-283 (1970).
34. Martin P. A. W. and Travers R. S., *Appl. Environ. Microbiol*., **55**(10): 2437-2442 (1989).
35. Masson L., Erlandson M., Puzstai-Carey M., Brousseau R., Juarez-Perez V. and Frutos R., *Appl. Environ. Microbiol*., **64**(12): 4782-4788 (1998).