5. DISCUSSION

Development and spread of antibiotic resistance in bacterial pathogens has been the driving force for scientists to seek alternatives. Several strains of human pathogens are known to contain up to 10 different genes coding for resistance (Henry, 2000). As bacteria continuously overcome the tools with which humans fight them, there is an urgent need to address the search for new products that affect the target. Great attention has been focused on bacteriophages, probiotics and antimicrobial peptides including bacteriocins (Asaduzzaman et al., 2009; Lin et al., 2009). Bacteriocins are ribosomally synthesized bacterial proteins or peptides with narrow or broad antimicrobial activity spectrum against other bacteria. They are produced by both Gram negative and Gram positive bacteria, and 99% of bacteria possess at least one bacteriocin in their repertoire for their microbial defense system (Klaenhammer, 1988).

Bacteriocins have a large degree of structural and biochemical diversity. Although a large numbers of bacteriocins have been identified and characterized, new bacteriocins are still being discovered and documented.

5.1 ISOLATION OF BACTERIOCIN PRODUCING BACTERIA FROM MARINE ENVIRONMENT

The present study was focused on the characterization of bacteriocins produced by bacteria isolated from the marine environment. With 70% of the earth’s surface being covered by the ocean and representing 80% of life on earth, this enormous pool of microbial biodiversity is under utilized and therefore retains the potential for discovery of new natural products (Kennedy et al., 2008). Isolation of bacteria can originate not only from sediments, but also from open oceans or marine surfaces including marine living organisms (Jensen and Fenical, 1994). Antibiotic production by marine bacteria has been documented for a long time.
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time (Rosenfeld & Zobell, 1947). Since these earlier reports, many low molecular weight antibiotic substances have been isolated from marine bacteria (Faulkner, 2001).

Of the one hundred and twelve isolates screened in the present study for their antibacterial property, only seventeen inhibited the growth of Gram positive/ Gram negative bacteria. After secondary screening using acetone precipitation and ammonium sulphate fractionation, the isolates were short listed to five that produced antibacterial compounds that were proteinaceous in nature. Marine microorganisms such as bacteria and fungi have been previously reported to produce antibacterial compounds. Competition among microbes for space and nutrient is a powerful selection pressure that endows marine microorganisms with the ability to produce natural products possessing industrial and medical values. De la Rosa-Garcia et al. (2007) screened 258 bacterial strains isolated from water and sediment in the Yucatan peninsula for antimicrobial activity and found 46 strains belonging to the genera *Aeromonas, Bacillus, Burkholderia, Photobacterium, Pseudomonas, Serratia* and *Stenotrophomonas* possessed antimicrobial activity. Approximately fifty percent of this antimicrobial activity was due to bacteriocins or bacteriocin like substances (BLIS). *B. subtilis* had also been isolated repeatedly from marine environments (Miranda et al., 2008; Ivanova et al., 1999).

Rosenfeld & Zobell (In 1947) carried out a detailed study on the antibiotic producing marine microorganisms. They found that most antibiotic-producing bacteria belonged to the genera *Bacillus* and *Micrococcus*. Although they did not attempt an isolation of specific antibiotics produced by marine bacteria, it was evident from their work that various species of microorganisms indigenous to the sea elaborate antimicrobial substances and they even suggested that the sea may represent a reservoir of microbial antagonists of possible importance.
5.2 CHARACTERIZATION OF BACTERIOCIN PRODUCING BACTERIA

The five bacteriocin producing isolates were identified using 16S ribotyping. The isolates were identified as bacilli belonging to two species, *B. subtilis* and *B. licheniformis*. 16S rRNA gene sequence analysis is a reliable method for identifying unknown bacterial isolates. The data generated using the universal 16S rRNA gene segment is of great accuracy and reproducibility. This method is less time consuming compared to the conventional phenotypic identification schemes. A 16S rRNA gene sequence similarity of ≥97% is a reasonable level for grouping bacteria into species (Hagstrom et al., 2000). Conventionally, bacilli have been identified in the laboratory through biochemical tests and fatty acid methyl ester profiling (Bobbie and White, 1980; Vaerewijck et al., 2001). These are technically complex and labour intensive procedures and the scarcity of reproducible and distinguishable phenotypic characteristics for several bacterial species often makes precise identifications difficult (Khamis et al., 2003). To date, the development of gene amplification and sequencing, especially that of the 16S rRNA gene sequences, has simplified the identification and the detection of specific bacteria, especially those lacking distinguishable phenotypic characteristics (Woese, 1987; Yamada et al., 1997; Kolbert and Persing, 1999; Shaver et al., 2002; Wang et al., 2003; Wu et al., 2006). The 16S rRNA gene is now used as a framework for the modern classification of bacteria, including those in the genus *Bacillus*.

Phylogram drawn with selected strains helped to study their relatedness or variability. Neighbour joining method was successfully used for this purpose. It was clear from the phylogram that the bacteriocin producing strains in the present study grouped together as clade. Within the clade they formed two groups, with the three *B. licheniformis* and the two *B. subtilis* strains grouping together, but
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separately from each other. The three *B. licheniformis* strains showed relatedness as did the two *B. subtilis* strains.

Among diverse microbial species, isolates of marine *Bacillus* belong to phylogenetically and phenogenetically heterogeneous groups of bacteria. They are ubiquitous in the marine environment and can tolerate adverse conditions such as high temperature, pressure, salinity, and pH (Rampelotto, 2010). Generally, *Bacillus* strains need more nutrition and space to be the fastest growing bacteria for which they compete with other organisms. Due to the diluting effect of the ocean drives, marine organisms produce potent bioactive compounds to fight off their competitors or to escape from micropredation (Paul et al., 2007). Metabolically marine strains are different from their terrestrial counterparts, and thereby they may produce unique bioactive compounds which are not found in their terrestrial counterparts (Jensen & Fenical, 1994). The ability to produce diverse classes of antibiotics by *Bacillus* sp. has been evident by several genomic studies. For example, the genome sequence of the widely distributed *Bacillus* strains revealed that about 8% of genome is devoted to synthesizing antibiotics (Kunst et al., 1997; Chen et al., 2007).

The two strains *B. subtilis* strain BTFK101 and *B. licheniformis* strain BTHT8 were selected for the further study. Ivanova et al. (1999) characterized twenty aerobic endospore-forming bacilli, isolated from marine invertebrates and sea water of different areas of the Pacific ocean. Most of the bacilli (11 strains) of marine origin belonged to the species *B. subtilis*, two strains were *B. licheniformis*, others were *B. amyloliquefaciens*, *B. pumilus*, *B. firmus*, *B. lentus*, *B. sphaericus*, *B. fusiformis*, *B. megaterium* and *Paenibacillus* sp. Although growth in marine water might occur, the abundance of *B. subtilis* in these environments might also be explained by its observed association with the gastrointestinal tract of marine organisms (Newaj-Fyzul et al., 2007).
Both strains, BTKF101 and BTHT8 inhibited the growth of only Gram positive organisms, majority of which belonged to genus Bacillus. They also inhibited *Cl. perfringens* and *S. aureus*. The inhibitory spectrum of bacteriocins can be narrow and confined to closely related species or it can be relatively broad, inhibiting a range of target organisms including food-spoilage and pathogenic bacteria such as *Listeria monocytogenes*, *B. cereus*, *Clostridium tyrobutyricum*, methicillin-resistant *S. aureus* and vancomycin-resistant enterococci (Cotter et al., 2005; Galvez et al., 2008). The activity range of bacillocin 490 was rather narrow and limited to some Gram-positive bacteria like *B. stearothermophilus*, *B. smithii*, *B. subtilis*, *B. anthracis*, *B. cereus*, *B. licheniformis*, *Listeria innocua* and *S. aureus* (Martirani et al., 2002). A bacteriocin like inhibitory substance (BLIS) with a broad spectrum of activity against pathogenic and spoilage bacteria (*L. monocytogenes*, *B. cereus* and clinical isolates of *Streptococcus* sp.) was produced by *B. licheniformis* P40 that was isolated from the Amazon basin (Cladera-Olivera et al., 2004). *B. licheniformis* strain ZJU12 isolated from soil produces a 3 kDa BLIS that has a broad spectrum of antagonistic activity against various species of Gram-positive bacteria, as well as pathogenic fungi (Lili et al., 2006). A low-molecular-weight peptide (1.5 kDa by SDS-PAGE) produced by *B. licheniformis* MKU3 (Kayalvizhi & Gunasekaran, 2008) exhibited bactericidal activity against Gram positive and Gram negative bacteria as well as different filamentous fungi and yeast (*Candida albicans*).

Subtilin produced by *B. subtilis* showed antimicrobial activity in the nanomolar range against a broad spectrum of Gram-positive bacteria (Gross et al., 1973). Ericins (mainly ericin S) are active against a variety of bacteria including the causative agent of tomato bacterial canker, *Clavibacter michiganensis* (Stein et al., 2002 a). Sublancin 168 produced by *B. subtilis* 168 exhibits bactericidal activity against other Gram-positive bacteria including important pathogens such
as *B. cereus*, *Streptococcus pyogenes* and *S. aureus* (Paik et al., 1998). Mersacidin produced by *B. subtilis* strain HILY-85, 54728 is a tetracyclic peptide active against methicillin and vancomycin-resistant *S. aureus* (Bierbaum et al., 1995). *B. subtilis* LFB112 from Chinese herbs produced a BLIS active against both Gram-positive and Gram-negative bacteria involved in domestic animal diseases including *E. coli*, *Salmonella Pullorum*, *P. aeruginosa*, *Pasteurella multocida*, *C. perfringens*, *M. luteus*, *Streptococcus bovis* and *S. aureus* (Xie et al., 2009). *B. subtilis* 14B isolated from the rhizosphere of healthy plants (bitter almond) produces a BLIS (Bac 14B) active against *Agrobacterium tumefaciens* (Hammami et al., 2009). *B. subtilis* strain MJP1 isolated from ‘meju’ (Korean fermented soybean) produces a BLIS with antimicrobial activity against various species of Gram-positive bacteria, yeasts and molds including food-spoilage microorganisms (Yang & Chang, 2007).

Antibacterial activity of the bacteriocins, BL8 and BS101 from the strains BTHT8 and BTFK101 respectively was tested against *S. aureus* in further studies. *S. aureus* has extensive genomic variability and easily acquires tools for resisting against antimicrobials, in particular against β-lactam antibiotics. Therefore it is one of the most successful and adaptable human pathogens (Daini and Akana, 2009). Methicillin resistant *S. aureus* (MRSA) has remained a major cause of nosocomial disease world-wide (Larsen et al., 2008).

As plasmid DNA was not obtained from the two strains BTFK101 and BTHT8, it was concluded that the genes encoding the bacteriocins are located on the chromosome. A survey of plasmid diversity among 50 natural isolates of *B. subtilis* estimated that only ~10% of strains harbour these extrachromosommal elements (Zawadzki et al., 1996). Lichenin is a chromosomally encoded bacteriocin (Pattnaik et al., 2005) produced under anaerobic conditions by *B. licheniformis* 26L-10/3RA isolated from water buffalo rumen (Pattnaik et al.,
Plasmid encoded bacteriocins are also reported in other species of *Bacillus*. Megacin BII is encoded on plasmid pSE 203 in strains of *B. megaterium* (Stahl, 1989). *Lysinibacillus sphaericus* SOPB1 was shown to produce a BLIS encoded by the pSOPB1-19 plasmid. *B. thuringiensis* HD-2 produces a plasmid-encoded thuricin HD2 (Favret & Yousten, 1989).

There are several species of *Bacillus* that are known pathogens. These include *B. anthracis* which is pathogenic to humans and other animals, and *B. cereus* which is a common cause of food poisoning (Claus and Berkeley, 1986; Norris et al., 1981). *B. subtilis* and *B. licheniformis* appear to have a low degree of virulence to humans. Although *B. subtilis* has been associated with outbreaks of food poisoning (Logan, 1988), the exact nature of its involvement has not been established. *B. licheniformis* can also cause food-borne gastro-enteritis. Dairy products are at increased risk of being contaminated with toxin-producing isolates of *B. licheniformis*. Cooked meats, raw milk, vegetables, and processed baby foods are also at risk. (Salkinoja-Salonen, 1999). de Boer et al. (1994) has documented in the review that authorities in the United States, Europe and Japan have approved production with and products from recombinant *B. licheniformis* strains and concluded that *B. licheniformis* is a safe host for the production of harmless, industrial products.

Hemolysis on blood agar plates is an indication of the potential of bacteria for pathogenesis. The two strains BTFK101 and BTHT8 tested on blood agar plate were non-hemolytic indicating their non-pathogenic nature. The non-pathogenic nature of the bacterium is one of the major criterions for selection of microbes for large scale production of any product (Byrom, 1992).

In the antibiotic susceptibility tests, both the strains were sensitive to the commonly used antibiotics like ciprofloxacin, gentamicin and trimethoprim. The strains which are sensitive to commonly used antibiotics can be regarded as safe
(De Vuyst et al., 2003) especially when they are intended to be incorporated in food products.

*B. subtilis* strain BTFK101 and *B. licheniformis* strain BTHT8 start their bacteriocin production at early exponential phase and the production reached its peak at the end of exponential phase/starting of stationary phase. Consequently, the bacteriocins were produced in the active growth phase. These characteristics of bacteriocin production clearly differentiate it from that of authentic secondary metabolites (Kleinkauf et al., 1986; Martin & Liras, 1989). The bacteriocin production in *Bacillus* strain 8A, reported by Bizani and Brandelli (2002) started at the exponential phase and reached its peak at the stationary phase. Cherif et al. (2001) reported that thuricin 7, produced by *B. thuringiensis* BMG1.7 expressed in the exponential growth phase. Production of bacteriocins in the exponential growth phase of bacteria is also reported in *B. subtilis* (Alam et al., 2011; Xie et al., 2009); in *B. licheniformis* (Cladera-Olivera et al., 2004; He et al., 2006) and in *B. brevis* (Saleem et al., 2009). Bacteriocins of lactic acid bacteria, particularly lantibiotics are usually produced in the exponential phase (Horner et al., 1990). Evaluation of bacteriocin production along with the growth curve was also studied by Torkar and Matijasic (2003) on strains of *B. cereus* 15/5, *B. cereus* 8/10, *B. cereus* 30/11 and *B. cereus* 8/2. In all the four strains, bacteriocin production and secretion was observed in the stationary phase (after 10 to 16 h) of bacterial growth. Naclerio et al. (1993) also reported the production and activity of bacteriocin cerein by *B. cereus* strain in the early stationary phase.

5.3 OPTIMIZATION OF BIOPROCESS VARIABLES BY ‘ONE-FACTOR AT-A-TIME’ METHOD

Eleven factors were taken into account viz. different media, sodium chloride (NaCl) concentration, carbon source, inorganic nitrogen source, organic nitrogen source, initial pH of the media, inoculum concentration, incubation
temperature, surfactant concentration, incubation period and agitation. Bacteriocin is usually produced in liquid substrates. The optimum conditions for bacteriocin production are affected by growth phase of the bacterium, pH of the medium, incubation temperature, type of carbon and nitrogen sources, and concentration of NaCl (Kim et al., 2000). Likewise, the bacteriocin production was enhanced by optimization of culture conditions in *L. casei* (Vignolo et al., 1995) and *Leuconostoc mesenteroides* (Krier et al., 1998).

Zobell marine broth was found to be the best media for bacteriocin production by both BTFK101 and BTHT8. Bacteriocin production was not attained when lactose broth was used for BTFK101. For BTHT8 also, bacteriocin production was not observed when lactose broth and Mueller-Hinton broth were used. The production of bacteriocin by *B. amyloliquefaciens* was studied by Lisboa et al. (2006) and maximum antibacterial activity was evaluated by cultivation in brain heart infusion (BHI). The effect of various media for the production of bacteriocin like inhibitory substance from microalgal symbiotic *Vibrio* sp. MMB2 was examined using media such as MRS, Luria broth, peptone enrichment media, peptic soya broth, TCBS, nutrient broth and Zobell marine broth (Selvendran and Michael, 2013). The maximum bacteriocin activity was recorded at MRS broth against *Vibrio harveyi*. Zobell marine broth stood next. The minimum bacteriocin activity was recorded in nutrient broth against *V. harveyi* and no activity was found when peptone enrichment broth and lactose broth were used.

While optimizing sodium chloride concentration, maximum bacteriocin production was obtained from BTFK101, when sodium chloride (NaCl) was supplied in concentrations ranging from 0.5 -2%. But specific activity was highest when 1.5% NaCl was provided. So 1.5% NaCl is optimum for BTFK101 for bacteriocin production. As the concentration of sodium chloride increased, the
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production of bacteriocins lowered. In the case of BTHT8, bacteriocin production reached its peak when NaCl was supplied at a concentration of 1%. The bacteriocin production completely stopped when NaCl was supplied at 2.5% or above. El- Sersy et al. (2009) has optimised the production of antimicrobial agents from B. licheniformis SN2 and found that the optimum NaCl concentration was 0.3%. The maximum bacteriocin production by Lactobacillus sp. MSU3IR was achieved with 3% NaCl supplementation. NaCl could alter the osmolarity of the cell membrane of bacterium which favoured more extrusion of bacteriocin from cell to media (El- Sersy et al., 2009). In correlation, Herranz et al. (2001) also reported that bacteriocin production by E. faceium P13 was high at 3% NaCl and more than 7% of NaCl supplementation reciprocally affected the bacteriocin production.

With strain BTFK101 maximum bacteriocin production was obtained when pectin was supplied as the carbon source. No production was seen when lactose was used as the carbon source. In the case of BTHT8, maximum specific activity was found for glucose although sucrose, starch and pectin gave same rate of production. So glucose can be considered as the best carbon source. There was no production of bacteriocin when lactose and mannitol were used. The role of different carbon sources on bacteriocin production by B. amyloliquifaciens was studied by Vijayalakshmi et al. (2011 a). Maximal activity was observed when glucose was used as carbon source. Fructose, maltose, glycerol, sucrose and xylose gave same rate of bacteriocin production followed by lactose and galactose. Mannitol supported moderate activity whereas inositol was a poor carbon source for bacterial growth. Similar results were reported by Ogunbanwo et al. (2003) where bacteriocin by L. brevis OG1 were synthesized in larger amounts in medium supplemented with 1.0% (w/v) glucose. Lactobacillus pentosus ST151 BR showed maximum activity when glucose was used at a concentration of 1.0% (w/v) in the production medium (Todorov and Dicks, 2004). The response surface
methodology was effective in optimizing bacteriocin-like substance production by
*B. cereus* XH25 (Zhong et al., 2013). Optimal bacteriocin activity was recognized
in *B. megaterium* 19 after 24 h of incubation in MRS supplemented with 4%
sucrose, followed by glucose, maltose, fructose and finally lactose. Galactose did
not stimulate the bacteriocin production (Khalil et al., 2009 a & b).

The optimization studies of nitrogen sources showed maximum
production when ammonium sulphate was used as inorganic nitrogen source for
both the strains. Production was lowest when urea was opted as the inorganic
nitrogen source. Beef extract proved to be the best organic nitrogen source for
both the strains. Lowest production was seen when malt was used. However the
effect of nitrogen source on bacteriocin production by Lactobacillus* sp. MSU3IR
revealed that, ammonium acetate favoured maximum bacteriocin production and
minimum bacteriocin production was noticed in sodium nitrate (El-Sersy *et al*.,
2009). Accordingly, the work of Iyapparaj *et al.* (2013) evidenced that the
increment in bacteriocin production was attributed with organic nitrogen source.
Cell growth and bacteriocin production was shown to be influenced by organic
nitrogen source (Kim *et al*., 1997).

Both the strains gave maximum production when inoculum at a
concentration of 6-10% was used. For BTFK101 specific activity was highest
when the media was inoculated with 10% culture and for BTHT8 it was 6%
inoculum. In both the strains, when inoculum below 5% was used, the production
reduced. Vijayalakshmi *et al.* (2011 a & B) studied on production of antimicrobial
protein from *B. amyloliquefaciens* MBL27 and found that growth and bacteriocin
production was minimal when 10^5 cells/mL was added. Bacterial growth and
production was maximal when 10^6 cells/mL was inoculated to the medium. When
the count was increased beyond this level, production and growth was decreased
which may be due to the exhaustion of nutrients.
**Discussion**

Tween 80 reduced the production in BTFK101. When tween 80 at a concentration up to 0.2% was supplemented in the media, the production remained the same as in the control but the specific activity decreased. Thus the surfactant has a negative impact on the production. But for BTHT8 the surfactant had a positive impact on the bacteriocin production. Addition of 0.1% tween 80 in the media doubled the production. But when the surfactant concentration was increased to 0.5%, the production decreased. Bacteriocin production by candidate bacterium emphasized that, the higher bacteriocin yield was attained in the medium supplied with tween 80 compared to other tested surfactants. Similar results were recorded for lactocin 705 (Vignolo et al., 1995). Possibly, tween 80 could change the surface tension of the producer cell thereby increasing the rate of bacteriocin release from the cell surface. Previous reports have shown an increased yield of bacteriocin production when tween 80 or tween 20 was added to the growth medium (Garver and Muriana, 1994; Huot et al., 1996; Ayemerich et al., 2000). Tween 80 increased the bacteriocin level by deadsorption of preformed bacteriocin and not by increasing its production or secretion, since the effect of the detergent was instantaneous. Non-ionic detergents such as tween 80 may mimic the effect of various food constituents inducing the production of bacteriocins and they are known to stimulate protein secretion by affecting membrane fluidity (Reese & Maguire, 1969). Tween-treated cultures also increased the supernatant activity relative to total activity, probably by desorption and disaggregation of the bacteriocin (Ayemerich et al., 2000; Mortevedt et al., 1991). Keren et al. (2004) determined the minimal tween 80 concentration required for maximal deadsorption of lacticin RM to be 0.025% and that it does not stimulate growth or bacteriocin production. They found that tween 80 reduced the adsorption of lacticin RM to producer cells and this effect is instantaneous, leaving no significant residual bacteriocin adsorbed to the cells.
Initial pH of the media had significant role in bacteriocin production by both the strains. pH 5-9 was found to be the optimum pH range for bacteriocin production by BTFK101. Specific activity was highest when media had pH 5. So media pH 5 is the best for the bacteriocin production by BTFK101. At pH 4 and 10, the production decreased. In the case of BTHT8, maximum production was seen when initial pH of the media was adjusted to 7. Production reduced when pH was lowered or raised from 7. El-Sersy et al. (2009) have optimised the media pH for the production of antimicrobial agents by B. licheniformis SN2 as pH 5. The initial pH of the medium has a profound effect on the production of bacteriocin. Kim et al. (2006) also reported that micrococcin GO5 production was maximum when the initial pH was between 7.0 and 9.0. At pH 4.0 the activity was slightly lower. Bacteriocin production by B. subtilis LFE-1, B.firmus H2O-1 and B.licheniformis T6-5 (Korenblum et al., 2005) was at an efficient level upto pH 10. But at pH 11, bacterial growth and bacteriocin production were. The pH of the medium affects the cell growth, hence influencing the bacteriocin production. Among the tested pH of the media in Lactobacillus sp., the maximum bacteriocin production in terms of antagonistic activity was recorded at pH 5 (Iyapparaj et al., 2013). However, further increase in pH found to mitigate the bacteriocin production. The minimum bacteriocin production was recorded at pH 9. De Vuyst and Vandamme (1992) found that nisin production at pH 6.8 showed an upward curvature and a sharp decrease in activity at the end of growth. Maximum production corresponded to maximum biomass concentration. Similar peaks in bacteriocin activity were obtained for helveticin J (Joerger and Klaenhammer 1986) and lactacin B (Barefoot & Klaenhammer 1984) at pH 6.0; mesenterocin 5 at pH 5.0 (Daba et al., 1993) or enterocin 1146 at pH 5.5-6.5 (Parente & Ricciardi, 1994). Similar patterns were observed among other lantibiotic-producing organisms like Staphylococcus epidermidis (Horner et al. 1989 & 1990).
The optimum incubation temperature for bacteriocin production by BTFK101 and BTHT8 was found to be 30°C. Production decreased as temperature of incubation was increased. Specific activity was also highest at 30°C. Galvez et al. (1993) recorded maximal bacteriocin production by *B. licheniformis* when incubated at 28°C. El- Sery et al. (2009) optimised the temperature for bacteriocin production by *B. licheniformis* SN2 as 30°C. The production of bacteriocin from *B. amyloliquefaciens*, studied by Lisboa et al. (2006) was maximum when incubated at 37°C. A similar level of activity was observed at 30°C but growth was negligible and no antimicrobial activity was observed at 55°C. Maximum production of bacteriocin was observed at 30°C in *B. amyloliquefaciens* (Vijayalakshmi et al., 2011 a & b). In many *Lactobacillus* sp. 30°C was found to be the best incubation temperature for maximum bacteriocin production as reported by Paynter et al. (1997), Ogunbanwo et al. (2003), Moonchai et al. (2005) etc. Decrease in bacteriocin production with increase in incubation temperature was observed by Iyapparaj et al. (2013).

In BTFK101, the bacteriocin production started when the culture was inoculated for 6 h but maximum production was attained at about 12 h. Production decreased as incubation period was increased. No bacteriocin production was noted after 30 h of incubation. In the case of BTHT8 also production started at 6 h of incubation but it took 18 h to reach its maximum. The production decreased and remained in a stationary mode after 30 h. Boe (1996) found that the growth of lactic acid bacteria increased logarithmically with the increase in incubation period. The increase in biomass of the bacteria was followed by a rise in bacteriocin production. When bacterial growth reached the stationary phase, bacteriocin production decreased. Galvez et al. (1993) recorded maximal bacteriocin production by *B. licheniformis* after 72 h of incubation. Production of antimicrobial protein by *B. amyloliquefaciens* MBL27 was studied by Vijayalakshmi et al. (2011 a & b) and observed that bacteriocin production was
growth associated and also biomass production was proportional to glucose utilization and it was maximal during the late exponential phase (24h), retaining its activity throughout the stationary phase.

Agitation plays a major role in bacteriocin production by both the strains. The bacteriocin production was very low when the culture was kept stationary. Effect of shake and still culture conditions for bacteriocin production in *B. amyloliquefaciens* MBL27 (Vijayalakshmi *et al.*, 2011 b) was studied by incubating the cultures under both shake and still culture conditions. Bacterial growth and antimicrobial activity was noticed in both the conditions but the rate of bacteriocin production under shake culture conditions was comparatively higher indicating the supportive role of oxygen in bacteriocin production.

**5.4 PURIFICATION OF THE BACTERIOCINS**

The purification process involved concentration and fractionation using ammonium sulphate precipitation of the crude culture supernatant. This was followed by gel filtration using sephadex G-25 column. Only the 30-60% fraction of ammonium sulphate precipitation showed activity. So, only this fraction was collected further. The activity was increased drastically after this concentration step. The specific activity also increased step by step during purification process. There was a five fold increase in specific activity of BS101 after concentration using ammonium sulphate. For BL8, the specific activity increased six fold after ammonium sulphate fractionation. BS101 was seven fold purified and BL8 was 29 fold purified after gel filtration chromatography.

In order to study the biological activities and chemical structures of bacteriocins, it is necessary to develop appropriate purification procedures. Bacteriocins produced by Gram-positive bacteria are the most often secreted into
the growth medium. Therefore, the purification preferably starts by a concentration step using the cell-free culture supernatant. Then, several additional steps, the most often chromatographic steps are necessary to achieve a significant purity (Sebei et al., 2007).

Bacteriocin like inhibitory substance from *B. subtilis* BS15 was successfully precipitated by 80% ammonium sulphate precipitation. The total protein was estimated before and after the precipitation and specific-activity determined which was found markedly increased after ammonium sulphate precipitation (Alam et al., 2011). 80% Ammonium sulphate saturation was used to precipitate bacteriocins by *B. thuringiensis* subsp. *entomocidus*, *B. cereus* and *B. subtilis* LFB112 (Cherif et al., 2008; Risoen et al., 2004; Xie et al., 2009) respectively. The antimicrobial substance produced by *B. amyloliquefaciens* MBL27 was precipitated readily by adding 40% saturation with about 98% recovery of the antimicrobial protein (Vijayalakshmi et al., 2011 a & b). Ammonium sulphate fractionation was used as the first step in purification protocol in *B. thuringiensis* (Ahern et al., 2003) and in *B. lentus* (Sharma et al., 2006).

Purification of bacteriocins includes combination of several strategies. Subtilosin A was purified using 65% ammonium sulphate, followed by reversed-phase HPLC (Shelburne et al., 2007). Concentration using chloroform followed by HPLC using C18 was the procedure followed by Joseph et al. (2013) for purifying bacteriocin from *B. subtilis*. Bac thuricin F4 was purified ammonium sulphate followed by C18 reverse phase HPLC (Kamoun et al., 2005). Butanol extraction followed by C18 reverse phase HPLC was used by Gray et al. (2006 b) for the purification of thuricin 17. Ammonium sulfate fractionation - carboxymethyl (CM) sephadex column chromatography - sephadex G-100 gel filtration were performed on the culture filtrate *Bacillus amyloliquefaciens* J4 for purifying
bacteriocin J4 (Lim et al., 2011). In purification of an antimicrobial peptide produced by a novel *Bacillus* sp. (Motta et al., 2007), concentration resulted in 57 fold purification and gel filtration made 80 fold increase of specific activity followed by ion exchange chromatography.

Bacillocin 490 from *B. licheniformis* was purified with a purification factor taking advantage of its ability to bind to the sensitive cells of *B. smithii* (Martirani et al., 2002). In the purification of cerein MRX1 hydrophobic interaction chromatography (HIC) was used efficiently to concentrate the antimicrobial activity from the culture supernatant. Subsequently, more than two-fold increase in specific activity was obtained in this step. However, cation exchange chromatography (CEX) resulted in a significant loss of activity. RP-HPLC was carried out as final purification step and the fold of purity was increased. The ability of cerein MRX1 to adsorb to cellular surfaces was probably because of its high hydrophobicity.

### 5.5 CHARACTERIZATION OF THE BACTERIOCINS

The SDS-PAGE analysis of the purified bacteriocins helped to indicate purification of the bacteriocins to homogeneity. The approximate molecular mass of the bacteriocins were estimated based on SDS-PAGE analysis and activity on the gel. Appearance of clearing region on the gel near the dye front, brought about by inhibition of the test organism revealed that the bacteriocins have a very low molecular mass. Moreover, the intact molecular mass of BS101 and BL8 was determined by MALDI-TOF MS as 3.37 kDa and 1.4 kDa respectively. Many bacteriocins are reported from *Bacillus* sp. with low molecular mass. Subtilin produced by *B. subtilis* has a molecular mass of 3319.56 Da as revealed by MALDI-TOF MS (Stein, 2008). Ericin S (3442 Da) and ericin A (2986 Da) are two related lantibiotics produced by *B. subtilis* A1/3 with strong similarities to subtilin (Stein et al., 2002 a). Sublancin 168, the lantibiotic produced by *B. subtilis*
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168 is having a molecular mass of 3877.78 Da (Paik et al., 1998). *B. subtilis* LFB112 from Chinese herbs produces a BLIS having 6.3 kDa, as estimated by SDS-PAGE (Xie et al., 2009). Bac 14B, a BLIS produced by *B. subtilis* 14B has an estimated molecular weight of 21 kDa by SDS-PAGE (Hammami et al., 2009). *B. subtilis* strain MJP1 isolated from meju (Korean fermented soybean) produces a BLIS whose molecular weight is determined as ~ 2.4 kDa by PAGE (Yang & Chang, 2007). Subtilosin A has a molecular mass of 3399.7 Da (Marx et al., 2001). The molecular weight of lichenin from *B. licheniformis* 26L-10/3RA, estimated by SDS-PAGE and gel filtration chromatography was approximately 1400–1500 Da. Bacillocin 490 produced by a thermophilic strain of *B. licheniformis* 490/5 isolated from a dairy product is a 2-kDa peptide (Martirani et al., 2002). A low-molecular-weight peptide (1.5 kDa by SDS-PAGE) is found to be produced by *B. licheniformis* MKU3 (Kayalvizhi & Gunasekaran, 2008).

Using isoelectric focusing (IEF), the isoelectric points of the bacteriocins were determined. The pI of BS101 was found to be 6.3 and that of BL8 was found to be 5.7. Many reported bacteriocins from Gram positive bacteria have a pI value greater than 7 (Ray, 1992; Hancock et al., 1995). Subtilosin A has a pI value of approximately 4, based on its amino acid sequence (Babasaki et al., 1985). Bacteriocin produced by a *B. subtilis* strain also has a pI of 4.7 (Zheng & Slavik, 1999). The isoelectric point of the bulgaricin appeared to be around 6.2 (Hasan et al., 2012).

N-terminal amino acid analysis of BL8 revealed the 13 amino acid sequence stretch. In amino acid position 4, 5 and 8 Edman degradation gave blank signals that are observed when cysteine residues are present (Yanagida et al., 2005). So these three positions are assumed to contain cysteine and the sequence is as follows: NH2-Ser-Trp-Ser-Cys-Cys-Gly-Asn-Cys-Ser-Ile-Ser-Gly-Ser-COOH. Comparison of the sequence with the amino acid sequence of bacteriocins
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in the NCBI protein database by multiple sequence alignment showed no significant similarity to any of the sequences, indicating the novelty of the bacteriocin. N-terminal sequencing of the thuricin 439A and B peptides by Edman degradation showed identical amino acid sequences. A sequence of 20 amino acids was obtained for each fraction: G-W-V-A- X-V-G-A-X-G-T-V-V-L-A-S-G- G-V-V (the X indicates an amino acid for which the identity could not conclusively be established; however, these two amino acids are likely to correspond to cysteine residues). The obtained sequence was compared to sequences present in the non redundant NCBI database. No significant similarity to any sequence was observed; indicating that thuricin 439 is a novel BLIS compound (Ahern et al., 2003). N-terminal amino acid sequencing was carried out for cerein 7A: NH$_2$- GWGDVL- and cerein 7B: NH$_2$- GWWNSWGH- by (Oscariz et al. (2006); cerein MRX1: NH$_2$- DWTCWCLVCAACSVELL by Sebei et al. (2007). The determined 18-amino acid sequence of cerein MRX1 is different from the sequences of cereins 7A and 7B but very similar to the 18-amino acid sequence of a recently discovered thuricin 17 produced by B. thuringiensis NEB17 (Gray et al., 2006 b). The difference between the two sequences resides in the residue in position 10 which is a cysteine in cerein MRX1 while it is a valine in thuricin 17.

The action of proteases viz. proteinase K, pepsin and trypsin on the bacteriocins was studied. There was complete loss/ reduction in activity of the bacteriocins when treated with proteases confirming their proteinaceous nature. Similar results are reported in bacteriocins of many Bacillus sp. B. subtilis LFB112 from chinese herbs produces a BLIS, sensitive to proteinase K and pronase E, but resistant to papain, trypsin and pepsin (Xie et al., 2009). Bac 14B, a BLIS produced by B. subtilis 14B was found sensitive to proteases. BLIS from B. amyloliquefaciens LBM 5006 (Lisboa et al., 2006); Brevicin AF01 from B. brevis AF01 (Faheem et al., 2007); Cerein 8A from B. cereus 8A (Bizani & Brandelli,
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BLIS produced by \textit{B. lentus} (Sharma \textit{et al.}, 2009a); Megacins19 (Khalil \textit{et al.}, 2009a); polyfermenticin SCD (Lee \textit{et al.}, 2001); thuricin CD (Hill \textit{et al.}, 2009); thuricin 7 (Cherif \textit{et al.}, 2001); thermoleovorins (Novotny & Perry, 1992); entomocin 9 (Cherif \textit{et al.}, 2003) and P45 by \textit{Bacillus} sp. P45 (Sirtori \textit{et al.}, 2006) all are reported as protease sensitive bacteriocins. But \textit{Bacillus firmus} H2O-1 (Korenblum \textit{et al.}, 2005); \textit{B. subtilis} strain MJP1 (Yang & Chang, 2007) and \textit{G. stearothermophilus} NU-10 (Fikes \textit{et al.}, 1983) produce bacteriocins resistant to proteolytic enzymes.

The bacteriocins BS101 and BL8 are able to tolerate wide pH range and temperature stresses. BS101 starts deactivating at 40°C and when it reaches 50°C it loses its activity completely, whereas BL8 was stable up to 60°C. Both BS101 and BL8 could tolerate a pH range of 2-9. Many bacteriocins are able to tolerate temperature and pH stresses. \textit{B. subtilis} LFB112 from Chinese herbs produces a BLIS that is heat and pH (3–10) stable (Xie \textit{et al.}, 2009). \textit{B. subtilis} 14B produces a BLIS (Bac 14B), stable to heat (up to 100°C for 2 h) (Hammami \textit{et al.}, 2009). \textit{B. subtilis} strain MJP1 produces a BLIS stable in the pH range of 6.0–10.0 and heat stable (Yang & Chang, 2007). Lichenin produced by \textit{B. licheniformis} 26L-10/3RA is heat stable, retaining biological activity even after boiling for 10 min and is active over a wide pH range of 4.0–9.0 (Pattnaik \textit{et al.}, 2001). Bacillocin 490 is produced by a thermophilic strain of \textit{B. licheniformis} 490/5 isolated from a dairy product, with a high stability at 4 and 100°C and stable over a wide pH range (Martirani \textit{et al.}, 2002). \textit{B. licheniformis} P40 that originated from the Amazon basin (Cladera-Olivera \textit{et al.}, 2004) produces a BLIS, stable over a very wide pH range (3–11) and at a high temperature of 100°C, but lost its activity at 121°C for 15 min.

In both BS101 and BL8, non-ionic detergents viz. tween 20, tween 80 and triton X-100 did not affect the activity of the bacteriocins. The ionic detergent
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SDS however reduced the activity of both BS101 and BL8. CTAB has very prominent effect. It completely deactivated both the bacteriocins. SDS inhibited the bacteriocin produced by *B. subtilis* LFB112 (Xie et al., 2009) but non ionic surfactants had no effect. Treatment with tween 80 increased antimicrobial activity whereas SDS and triton X-100 had no effect on the bacteriocin activity of bacteriocin G2 produced by the probiotic bacteria *Lactobacillus plantarum* G2 (Seatovic et al., 2011). Surfactants did not affect the bacteriocin or bacteriocin like substance produced by *Lactococcus lactis* B14 (Ivanova et al., 2000). The antagonistic activity was greatly reduced when treated with SDS whereas triton X-100 and tween 20 completely inhibited the activity of plantaricin OL15 a bacteriocin produced by *Lactobacillus plantarum* OL15 (Mourad et al., 2005). It was observed that tween 80 adversely affected the activity of bacteriocin from *Leuconostoc* NT-1 (Maurya & Thakur, 2012). It was noticed that tween 80 and SDS increased the activity of the bacteriocin whereas Triton X-100 lowered the activity of plantaricin SR18 produced by *Lactobacillus plantarum* SR18 (El-Shouny et al., 2013). SDS, tween 80 and triton X-100 stimulated the bacteriocin activity of *Lactobacillus lactis* (Rajaram et al., 2010).

BS101 remained stable when it was exposed up to 8 mM DTT. Above this concentration, the activity reduced drastically. β - mercaptoethanol did not affect the activity of BS101 up to a concentration of 60 mM but the activity reduced when it was exposed to β-mercaptoethanol at a concentration of 70 mM and above. The activity of BL8 was reduced to half when it was exposed to 20 mM and above. The activity of BL8 was reduced when treated with β- mercaptoethanol at a concentration of 200 mM. A few reports are available on the study of the effect of reducing agents on bacteriocins. DTT reduced the activity of the bacteriocin produced by *B. subtilis* LFB112 (Xie et al., 2009). It was noticed that β-mercaptoethanol (0.2%) increased the activity of bacteriocin produced by *Lactobacillus plantarum* SR18 (El-Shouny, 2013) to 1.2-1.4 fold. Oxidation can
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occur when proteins are exposed to oxidizing agents such as hydrogen peroxide. DMSO is used as a mild oxidant. Oxidation of methionine residues is common and it has been shown to cause a decline in the biological activity of the protein. No reduction in activity of the bacteriocins was observed when treated with DMSO, which may be due to the absence of methionine residues in the bacteriocins. In the case of BL8 this is supported by N-terminal sequencing.

In the present study the ions of Na, Ca, Mg, Fe, Mn, Ni, Ba, Cd, Zn, Cu, Co and Al were evaluated for their impact on the activity of bacteriocins. For BS101 ions of metals like Mg, Al, Fe, Zn, Na, Cu, Ni, Ca, Mn and Co ions did not affect the stability and activity. The ions of two metals Ba and Cd increased the activity of BS101. They acted as activity promoters for BS101. For BL8 ions of Mg, Al and Ba increased the activity. Mn and Co ions decreased the activity. The metal ions of Cd, Fe, Zn, Na, Cu, Ni, and Ca had no role on the activity of the bacteriocin. Studies have indicated that metal ions impart thermodynamic stabilization to the native state of the protein by binding to the active site of the enzyme (Wyman and Gill, 1990). Ca ions have been reported to bind to the inner surface and autolytic sites of protein molecules thereby strengthening the interaction inside the molecule (Ghorbel et al., 2003).

The action of bacteriocins was found to be bactericidal for *S. aureus*, *B. circulans*, *B. coagulans* and *B. cereus*. But both the bacteriocins were bacteriostatic for *B. pumilus* and *Cl. perfringens*. Most bacteriocins exert bactericidal mode of action against the sensitive microorganisms, although some of the bacteriocins have been shown to act in a bacteriostatic manner. Most of bacteriocins from *Bacillus* sp. showed a bactericidal effect (Naclerio et al., 1993; Hyronimus et al., 1998; Cherif et al., 2003; Gray et al., 2006 b; Sharma et al., 2006; Aunpad and Na- Bangchang, 2007). Bacteriocin from *Lactococcus lactis* (Ivanova et al., 2000) was found to be bacteriostatic.
5.6 APPLICATION STUDIES

It was experimentally proved that both the bacteriocins perform very well in the control of biofilm formed by *B. cereus* and *Cl. perfringens*. *Bacillus* sp. particularly *B. cereus*, are implicated in food spoilage (Andersson *et al.*, 1995; Janneke *et al.*, 2007). Sharma & Anand (2002) studied the biofilm constitutive microflora in a commercial dairy plant and found that *B. cereus* accounted for more than 12% of the microflora. As *B. cereus* is ubiquitously present in nature, it is easily spread through food production systems and contamination with this species is almost inevitable. Moreover, *B. cereus* spores are both highly resistant to a large number of stresses and very hydrophobic, which causes them to adhere easily to food processing equipment (Lindsay *et al.*, 2006). *Cl. perfringens* is also one of the most ubiquitous bacteria in natural environments. Biofilm formation by *Cl. perfringens* has been reported by John *et al.* (2008). Bacteriocins are gaining importance and have a unique potential in the food industry for the effective biocontrol and removal of biofilms. These newer biocontrol strategies are considered important for the maintenance of biofilm-free systems and thus improve the quality and safety of foods. Bacteriocins have several characteristics that make them ideal food preservatives.

Both the bacteriocins proved to be very efficient in controlling the microflora of sea foods like prawns and anchovies. Taylor *et al.* (1990) studied the application of nisin A in the preservation of fish products. The cod, herring, and smoked mackerel fillets inoculated with *Cl. botulinum* spores brought about a delay in toxin production when they were treated with nisin. The bacterial growth of brined shrimp was put to a control and shelf life extended when treated with with nisin Z, carnocin U149 and bavaricin (Einarsson & Lauzon, 1995). Such results offer clear perspectives for the biopreservation of certain fish products with bacteriocins.
In order to study the effectiveness of bacteriocins in combating the bacterial pathogen in vivo, C. elegans was chosen as the model organism. C. elegans is a very convenient whole organism model to identify or assay antimicrobial compounds (Ewbank & Zugasti, 2011). It is a small, free-living soil nematode that feeds primarily on bacteria. Its usefulness as a model organism is due to its genetic tractability, rapid generation time, ease of propagation, transparent body, a well-defined cell lineage map and a fully sequenced genome that contains a large number of vertebrate orthologues (Portal-Celhay & Blaser, 2012). Several human pathogens including Pseudomonas aeruginosa, Salmonella Typhimurium, Serratia marcescens, Staphylococcus aureus, Vibrio cholerae, and Burkholderia pseudomallei kill C. elegans when supplied as a food source (Tan et al., 1999; Aballay et al., 2000; Kurz & Ewbank, 2000; Labrousse et al., 2000).

Both S. aureus and B. circulans were able to infect C. elegans and reduce the TD_{50}. S. aureus is a common Gram-positive bacterium that causes a range of minor infections, which occasionally become serious in many animals (Lindsay, 2010). In C. elegans, intact bacteria accumulate in the gut of the animal and it is this colonisation that eventually overwhelms the host, disrupting the gut epithelium and then destroying internal organs ultimately leading to death (Garsin et al., 2001; Irazoqui et al., 2010).

The ability of the bacteriocin BL8 to confer protection against the bacterial infection by S. aureus and B. circulans were proved by this study. It was observed that the bacteriocin BL8 succeeded in controlling the infection of C. elegans and extent the life span of the worms when compared to the untreated (control) worms fed on E. coli OP50. In vivo assays of many antimicrobial agents have been studied using C. elegans. Compounds that block quorum sensing were identified using C. elegans and potentially developed as antimicrobial drugs (Swem et al., 2009). Uccelletti et al. (2010) studied the antibiotic action of
membrane-active cationic antimicrobial peptides (CAMPs) from frog skin in *C. elegans*. Moy et al. (2006) developed a liquid-based assay using microtiter plates to test the potential of thousands of synthetic compounds and natural extracts to cure *C. elegans* following *Enterococcus faecalis* infection. Similar high-throughput in vivo assays have been used to screen for antimicrobials that are effective against other human pathogens, including the fungus *Candida albicans* (Breger et al., 2007; Okoli et al., 2009). These assays also provided the opportunity to assess the relative MIC, the effective concentration in vivo as well as the toxicity of these compounds in a single assay.

This work was envisaged to understand the potential of bacteriocins as biocontrol agents. During the course of this study, two potent bacteriocins, BS101 and BL8 capable of inhibiting the growth of many Gram positive pathogenic bacteria including *S. aureus* and *Cl. perfringens* were characterised. These small molecular weight peptides exhibited excellent properties like high stability and sturdiness under various physical and chemical exposures.