Chapter 6

SUMMARY

Multi-pond solar salterns are used worldwide for salt production along tropical and subtropical coastal areas and characteristic salt-adapted microbial communities are found along the salinity gradient in these saltern ponds. Further, in spite of the fact that halocin production is widespread among the *Halobacteriaceae*, no information is available on their ecological significance. Whereas, halocins may play a role in the interspecies competition between different types of halobacteria in saltern crystallizer ponds inhabited by dense communities of these red halophiles.

Research investigations pertaining to microbiology of saltern ponds of South India which contribute to significant amount of commercial salt production has not been made adequately. In this context the present investigation addressed the prospects of deriving haloarchaeal bacteria which may return potential halocins that may hold scope for applications in several industries.

A potential halocin producing haloarchaeal strain *Natrinema* sp BTSH10 and an indicator strain *Halorubrum* sp. BTSH03 were isolated from salt pan of Kanyakumari, Tamilnadu, India. They were identified based on polyphasic taxonomy characteristics. The 16S rRNA partial gene sequences were submitted to GenBank (JN228202-*Natrinema* sp.BTSH10, JF830242-*Halorubrum* sp.BTSH03) through Bioedit programme, at NCBI site. From the phylogenetic tree constructed, it was inferred that the *Natrinema* sp. is found in a separate branch indicating that this might
be a new strain which has to be further characterized and confirmed. *Halorubrum* sp. showed very close similarity with an uncultured archean and *Halorubrum xinjiangense*.

The halocin produced by *Natrinema* sp.BTSH10 was named as halocin SH10 after the name of the producing organism. The activity of the halocin against the sensitive strain *Halorubrum* sp.BTSH03 was confirmed by agar overlay method.

Antibiotic sensitivity studies were done using 31 antibiotics and *Natrinema* sp. showed multi drug resistance and the MAR index was calculated as 0.3870. *Halorubrum* sp. showed resistance to 3 antibiotics and its MAR index was calculated as 0.09677.

Lipid analysis of *Natrinema* sp. showed presence of 10 different lipids out of which only four were identifiable and remaining 6 were unidentified. Fatty acid methyl esterase (FAME) analysis showed the presence of many unidentifiable fatty acids.

*Natrinema* sp. BTSH10 formed halites which were of different sizes varying from few millimeters to upto 2cm length. SEM confirmed the colonization of these haloarchaea inside the crystal. ICP-AES analysis showed the presence of Fe, K, Mg, Na, Sr in the halite. Powder XRD studies, FTIR analysis, UV-VIS-NIR analysis and thermal analysis confirmed the crystallinity of the sample and also the thermal stability of the crystal.
Various bioprocess variables were optimized for halocin production by *Natrinema* sp.BTSH10. The initial medium used for production was Zobell’s broth. Strategy adopted for optimization was ‘one-factor-at-a-time’ method where the optimized variable will be constant in the forthcoming optimizations.

Optimization of incubation temperature for halocin showed that maximum halocin production was observed at 42°C which showed 1024AU of halocin. 37°C and 47°C also showed enhance in halocin production but only at lower levels.

Halocin production was observed over a broad pH range from pH 5 to pH 9 although maximum halocin production was recorded at 8.0 (1024AU). However, the bacteria did not produce halocin under acidic conditions.

Sodium chloride concentration was considered to be a key factor for halocin production. *Natrinema* sp. BTSH10 required 3M NaCl for maximum (1024AU) halocin production. NaCl concentration ranging from 2.5M – 4M (512AU) in the medium also supported enhanced production of halocin. *Natrinema* sp. BTSH10 required a minimum of 1.5M NaCl for its survival in the medium.

Among the different carbon sources used maximal halocin production was supported by the medium supplemented with galactose (2048AU) followed by sorbitol, maltose, glycerol, glucose (1024AU) and fructose, lactose (512AU). Whereas, medium supplemented with dextrin, sucrose and xylose supported reduced levels of halocin production. Galactose was observed to enhanced halocin production in the medium compared to other carbon sources.
Summary

Among the different nitrogen sources tested, the presence of beef extract (2048AU) in the medium supported maximal halocin by bacteria followed by soybean meal, malt extract, tryptone, peptone, yeast extract (1024AU) and casein, gelatin (512AU). Urea did not support halocin production.

Among the different inorganic salts used in halocin producing medium calcium chloride (2048AU) was found to support maximal halocin production by bacteria followed by magnesium chloride, sodium fluoride, potassium chloride, and sodium bicarbonate (1024AU). Whereas aluminium nitrate, potassium bromide, strontium chloride and sodium silicate led to a much reduced level of halocin production.

Agitation influenced halocin production. Maximum halocin production (4096AU) was observed at 200 rpm and 250 rpm. Lower agitation rates 50 rpm to 150 rpm also supported considerable levels of halocin production.

The time course experiment was conducted over a total period of 144h. Maximum (8192AU) production of halocin was observed at 104h, during the stationary phase. Halocin production was observed at considerable levels even after 88, 96, 112 and 128h where the halocin production was noted to be 4096AU. The halocin activity was found to be 2048AU after incubation for 80 and 144h.

Halocin SH10 was purified employing standard purification protocols which included ethanol precipitation of sample followed by molecular weight cut off centrifugation by 30kDa cut off membrane and gel filtration chromatography.
Fraction with halocin activity obtained after separation with molecular cut-off membrane confirmed that the molecular mass of the halocin is below 30kDa.

Sephadex G50 column was used for gel filtration chromatography and the elution was done using 50mM Tris-HCl buffer pH 8.0. A single peak was obtained for the elutants having halocin activity.

The purity of the halocin was confirmed by HPLC. The crude supernatant, 30kDa cut off centrifugation subjected supernatant and the gel filtration elute showed a single peak exactly between 11 and 12 min where the ACN/WATER volume was calculated as 32% : 68%.

Tricine PAGE was performed to confirm the purity of the halocin and determine its molecular mass. The molecular mass of halocin was calculated as 20kDa.

Bioautography assay was also performed on the unstained protein gel (Tricine PAGE) by agar overlay method which showed a clearing zone exactly at the 20kDa region comparing to the stained gel.

Cell lysis assay performed using halocin against Halorubrum sp.BTSH03 showed cell shrinkage and formation of small islands /colonies after 3h of incubation. Cell bulging and signs of cell lysis appeared after 6h and complete cell lysis after 12h of treatment.
Summary

N-Terminal sequencing enabled detection of first five amino acid which was found to be APFYI. MALDI and MASCOT analysis of the 20kDa halocin SH10 showed that it has a very close sequence similarity with 50S ribosomal protein L3P of *Methanosarcina acetivorans* (strain ATCC 35395).

NMR spectrum showed that halocin SH10 has excess of aliphatic chain containing aminoacids which are generally non polar and Hydrophobic. Total aminoacid analysis showed the presence of glycine (12.74%), asparagine (10.35%), glutamine (9.78%), valine (6.05%), alanine (3.94%), serine (3.69%), histidine (3.43%), threonine (3.11%) which confirms the NMR spectrum.

The halocin SH10 was thermostable up to 40°C (4096AU) without any loss of activity which however got declined to 1024AU at 60°C and 70°C and 128AU at 80°C and then lost activity at higher temperatures.

Halocin SH10 was stable at pH 6.0-8.0 (4096AU) and at pH 9.0 (2048AU) the activity got declined. At pH below 6.0 there was a rapid decrease in halocin activity.

The halocin SH10 retained its activity (4096AU) when incubated with 10% of organic solvents, upon incubation with 20% and 30% solvent halocin retained its activity with acetone, acetonitrile and isopropanol but lost activity with chloroform, methanol and ethanol after one hour incubation.

Halocin SH10 was found to have greater importance in leather industry. Halocin treatment for 12h could inhibit halophilic bacterial growth on hides. In raw
hides 67 ± 2 CFU were obtained and after treating with halocin for 3, 6 and 12h, CFU decreased considerably.

Halocin SH10 also found to have anticancerous activity which was confirmed by short time cell cytotoxicity studies conducted on DLA cells where the CTC₅₀ value was found to be 256AU. MTT assay carried out on cell lines to study anticancer activity of halocin in vitro, which showed that it had IC₅₀ value for HBL100 cell line was 4096AU which clearly indicated that it does not have a toxicity effect towards normal cell line. Cell cytotoxicity was shown towards other cancer cell lines used and IC₅₀ values were calculated as 1024AU for HeLa cell lines and 512AU for A549, OAW42 and HEp 2 cell lines. Accordingly 1024AU and 512AU concentration was used for in vivo anticancer study.

Halocin SH10 when treated towards DLA bearing mice showed that the parameter reverted to near normal comparing to normal DLA and DLA+5FU with the halocin SH10. This reversion is indicated by reduction in the body weight (grams) to 20.85 ± 1.73 after treatment of DLA mice from 34.23 ± 0.62 with halocin which may be a reason for the decrease in tumor cells. In the same way the increase in WBC (1x10³ mm³) from 10.41 ± 0.46 in normal to 19.58 ± 1.22 in DLA mice was reduced to 13.36 ± 0.45. RBC (1x10⁶ mm³) level also decreased to 5.52 ± 0.47 in DLA mice which was 11.10 ± 0.38 in normal mice and the level increased to 8.75 ± 0.16 upon treatment with halocin SH10. Hemoglobin (g/dl) level increased to 12.35 ± 0.72 upon treatment of DLA mice bearing 8.64 ± 0.68 which was lesser in content comparing to normal mice (11.10 ± 0.38). The differential WBC count also showed reversal of cell number near equal to normal mice after treatment with halocin SH10.
Chapter 7

CONCLUSION

Based on the results obtained in the present study it is concluded that haloarchaea holds a treasure of bioactive substances, particularly halocins which have immense scope for several applications. To the best of our knowledge this is the first halocin reported for *Natrinema* sp. Further this is the first time a halocin from a halocbacteria *Natrinema* sp. is shown to have anticancerous activity against major cancer cells concerned with humans. The results obtained for the characterization of this halocin showed that the halocin is stable at wide range of pH, organic solvents besides being thermostable. These characteristics add more importance to this halocin and indicate scope for other applications as a preservative in food and in leather industries. This halocin SH10 can also be used in leather industry for storing the leather, free from bacterial community which damages the hide and decreases the quality of the hide. There is ample scope for further research to be conducted on halocin such as biochemical characteristics, elucidation of structure.

This study on halocin production by *Natrinema* sp.BTSH10 indicate the prospects for intensive research which could lead to discovery of novel halocins which could have far reaching impact in biopharmaceutical industry particularly as anticancer drug. It is also anticipated that further research on this halocin could lead towards development of novel anticancer drug and new era in pharmaceutical biotechnology. There is no doubt that haloarchaea from saltern ponds have immense potential to return novel and valuable drugs and bioactive substances.