Summary

The reader should come away convinced from this thesis that you actually did learn something.

- Richard Brooks

Doing your best is more important than being the best.
The brine samples collected from salt pans of Kandla (Gujarat, India) and Bhayandar (Maharashtra, India) were found to be thalassohaline with the sodium and chloride ions being dominant and pH around 6.0.

Thirteen pink coloured isolates were obtained using membrane filter technique on medium containing 25% (w/v) NaCl. All the isolates were Gram negative and motile.

Minimum of 10-20% NaCl was required for growth of the isolates with optimum growth in presence of 25% NaCl confirming their halophilic nature.

Innsensitivity of isolates to antibiotics targeting peptidoglycan layer and sensitivity to bacitracin and novobiocin suggested their archaeal characteristics. Isolates showed varied biochemical properties. Anaerobic growth in presence of arginine and no acid production in presence of sugars by the isolates SP1(1), SP1(2b) and SP3(2) indicated their relatedness to the genus *Halobacterium*.

The isolates were further grouped based on their membrane polar lipid signatures. Isolates SP1(1), SP1(2b) and SP3(2) had polar lipid signature similar to *Halobacterium* sp. Polar lipid profiles of isolates SP1(2a), SP2(3), SP3(1), SOP, BS1, BS2a and BS2b were similar to that of *Haloferax* sp. whereas, isolates SP2(1), SP2(2) and SP4 possessed the lipid signature similar to *Haloarcula* sp. All the three genera belong to the family Halobacteriaceae of the domain Archaea.

The archaeal similarity of isolates was also examined by 16S rDNA restriction analysis (ARDRA). The results of restriction digestion pattern of 16S rDNA of isolates showed correlation with the results of their respective polar lipid profiles suggesting the existence of three different genera *Halobacterium*, *Haloferax* and *Haloarcula*.

The analysis of 16S rDNA sequences of isolates confirmed their archaeal identity. Isolates SP1(1), SP1(2b) and SP3(2) showed 98-99% similarity with *Halobacterium salinarum*; SP2(1), SP2(2), and SP4 exhibited 98-99% relatedness to *Haloarcula hispanica*; Sp1(2a) (97% similarity), SP2(3) (97% similarity) and SP3(1) (98% similarity) to *Haloferax mediterranei/Haloferax luctentense*; SOP and BS1 (99% similarity) to *Haloferax volcanii*; BS2a (100%) to *Haloferax alexandrinus*; and BS2b (99%) to *Haloferax volcanii/Haloferax alexandrinus*. These results were in agreement with the results of polar lipid analysis and ARDRA. The sequences have been submitted to NCBI GeneBank database.
The haloarchaeal isolates were examined for their extracellular protease producing ability on skimmed milk agar plates. *Halobacterium* sp. SP1(1), SP1(2b) and SP3(2) exhibited significant zone of clearance on skimmed milk agar indicating their potential for producing protease. The protease production by these isolates was estimated in soybean meal-based production medium at 37°C. Maximum protease production (18.16 ± 1.55 units/ml) was observed at 72 h by *Halobacterium* sp. SP1(1). Therefore, *Halobacterium* sp. SP1(1) was selected for further studies.

Effect of NaCl concentration on protease production by *Halobacterium* sp. SP1(1) was studied at 20, 25, 30 and 35% (w/v) NaCl. Significant protease production was observed at and beyond 25% NaCl suggesting this as the minimum required concentration for protease production.

Influence of various nutrients such as carbon and nitrogen source, metal ions, surfactants and amino acids on production of protease was investigated. Maximum protease production (42.23 units/ml) was obtained at 96 h in medium containing 1% (10 g/l) soybean flour which was 2.36 folds higher compared to the initial, soybean meal-based production medium. Further studies were conducted using soybean flour instead of soybean meal in the production medium. Significant increase (1.54 fold) in protease production was observed on addition of FeCl₃ (1 mM) to soybean flour-based production medium. Increase in protease production by 1.26 fold was observed in presence of surfactant dicotylsulfosuccinate (0.2 g/l). SDS (sodium dodecyl sulfate) at same concentration reduced the production by 2.3 fold whereas, CTAB (Cetyl trimethyl ammonium bromide) and Na-taurocholate inhibited growth of the isolate and hence, protease production. All the three amino acids (10 mM) tested (Na-glutamate, L-valine and L-alanine) inhibited the production to varied levels, with maximum inhibition (1.93 fold) caused by L-alanine.

Further screening of medium components was done by statistical approach using Plackett-Burman method. Soybean flour and FeCl₃ were identified as essential medium components having significant influence on protease production with a confidence level of 99.87 and 93.2%. Response surface methodology was applied for optimization of these components using central composite design (CCD). The optimized medium contained (g/l): NaCl, 250; KCl, 2; MgSO₄, 10; tri-Na-citrate, 1.5; soybean flour, 10 and FeCl₃, 0.16.
Extracellular protease from *Halobacterium* sp. SP1(1) was purified by gel permeation chromatography using sephacryl S-200 column. The protease was purified by 111.25 fold with a final yield of 17.41% and specific activity 520.25 U/mg protein. The molecular mass of purified protease as revealed by SDS PAGE analysis was found to be 42.1 KDa.

The purified protease hydrolysed casein and azocasein with specific activity 646.91 ± 9.04 and 198.2 ± 4.9, respectively. No activity was observed in presence of elastase (Suc-Ala-Ala-Ala-pNA) and esterase (paranitrophenyl acetate) specific substrates.

The optimum NaCl concentration, pH and temperature for protease activity were found to be 2 M, pH 7-9 and 30-50°C, respectively.

Among various chemicals (inhibitors, detergents and metal ions) tested, PMSF (a serine protease inhibitor) completely inhibited the protease activity while, CTAB and SDS inhibited the enzyme activity by 56 and 38%, respectively. None of the other chemicals tested had any significant effect on enzyme activity. All the osmolytes tested were able to support enzyme activity in absence of NaCl, 30% Na-glutamate showing maximal activity (189.86%)

The enzyme was highly active at different temperatures in presence of 30% Na-glutamate and exhibited a broad temperature range 20-80°C for activity.

The thermal stability of enzyme was highest in presence of 30% Na-glutamate followed by 4 M NaCl and 2 M NaCl. The \( t_{1/2} \) values of protease at 60°C in presence of 30% Na-glutamate, 4 M NaCl and 2 M NaCl were 438.7 min, 89.25 min and 14.36 min respectively. The enzyme was stable even at 80°C in presence of 30% Na-glutamate with \( t_{1/2} \) of 76.49 min.

The thermodynamic parameters for protease deactivation were calculated. The activation energies (\( E \)) for protease deactivation in presence of 30% Na-glutamate, 4 M NaCl and 2 M NaCl were 38.02 KJ/mole, 40.43 KJ/mole and 110.81 KJ/mole, respectively. Change in enthalpy (\( \Delta H^* \)) and entropy (\( \Delta S^* \)) for deactivation of protease in presence of 30% Na-glutamate, 4 M NaCl and 2 M NaCl were found to be 35.34 KJ/mole, -193.55 J/mole; 37.75 KJ/mole, -172.25 J/mole and 108.12 KJ/mole, 55.2 J/mole, respectively. The change in free energy (\( \Delta G^* \)) for protease deactivation at 60°C in presence of Na-glutamate, 4 M NaCl and 2 M NaCl were 99.76 KJ/mole; 95.35 KJ/mole and 90.29 KJ/mole, respectively. The low values of
enthalpy and entropy and high free energy values in presence of Na-glutamate signified the thermostability of protease.

The catalytic constants $K_m$ and $V_{max}$ for casein hydrolysis in presence of 2 M NaCl and 30% Na-glutamate at 37°C and pH 7.2 were 0.262 mg/ml; 40.984 U/ml and 1.266 mg/ml; 106.4 U/ml, respectively. The turnover number and second order rate constant for the enzyme in presence of 2 M NaCl and 30% Na-glutamate were 22.33 s$^{-1}$; 85.13 mg$^{-1}$ s$^{-1}$; 58.1 s$^{-1}$; 45.89 mg$^{-1}$ s$^{-1}$.

Studies were conducted to explore the potential of extracellular protease producing *Halobacterium* sp. SP1(1) for accelerating fish sauce fermentation as the traditional process takes 6-18 months for completion. Initial screening for fish protein hydrolysis was done using three different edible fishes found in Gujarat; pomfret, flat fish and seer fish in dry powdered form. Maximum protease production ($17.8 \pm 0.17$ U ml$^{-1}$) by *Halobacterium* sp. SP1(1) was observed at 48 h of growth in the medium containing pomfret powder. Optimum NaCl concentration for protease production in presence of pomfret powder was found to be 25%, w/v.

Traditional fish sauce fermentation process was mimicked using fresh white pomfret and 25% NaCl concentration (i.e. 4:1 ratio) in the system. The sauce samples were analysed for protease activity, peptide release and $\alpha$-amino content at regular interval of 10 days. These three parameters reached their maximum levels 10 days earlier in eviscerated and uneviscerated tests inoculated with *Halobacterium* sp. SP1(1) compared to the respective uninoculated controls. The total protein and nitrogen contents were high in both the tests compared to controls. The percentage of free amino acid content (69.64-63.39%) of the treatments (eviscerated test, eviscerated control and uneviscerated test) was comparable with that of commercial sauce (61.55%) except for uneviscerated control (40.37%). The taste active amino acids, aspartic acid and glutamic acid as well as the essential amino acid lysine in the experimentally fermented sauce (inoculated tests) reached to levels comparable with commercial sauce within 20 days. The microflora analysis of the final fish sauce revealed presence of extremely halophilie bacteria F1 and F2 in eviscerated and uneviscerated controls, respectively. These isolates were identified as *Halobacterium* sp. by 16S rDNA sequence analysis and the sequences were submitted to NCBI GeneBank database.
Effect of organic solvents on growth, protease production and protease activity of *Halobacterium* sp. SP1(1) was studied for exploring its possible solvent based applications. Organic solvent tolerance of *Halobacterium* sp. SP1(1) was found to be more compared to the other representatives SP1(2a) and SP2(2) of the genus *Haloferax* and *Haloarcula*.

Increase in NaCl concentration (10-30%, w/v) was found to increase the tolerance of isolates in presence of aliphatic hydrocarbons: n-dodecane (log $P_{ow} = 7.0$), n-undecane (log $P_{ow} = 6.5$) and n-decane (log $P_{ow} = 5.5$). No growth was observed in presence of xylene (log $P_{ow} = 3.1$) and toluene (log $P_{ow} = 2.8$) at any salt concentration. Solvents with log $P_{ow} < 4.5$ are considered to have harsh effects on cell membrane of organisms.

The effect of different concentrations of other metal ions Mg$^{+2}$, Ca$^{+2}$ and K$^+$ in combination of different NaCl concentrations (which are known to stabilize the cell membrane of bacteria/archaea) was, therefore, examined on the tolerance of *Halobacterium* sp. SP1(1) towards xylene and toluene. None of these metal ions could support the growth of isolates against these solvents suggesting that the isolate was actually sensitive to xylene and toluene.

Protease production by *Halobacterium* sp. SP1(1) in presence of n-dodecane, n-undecane and n-decane increased with decrease in log $P_{ow}$ value of solvents suggesting the possible effect of these solvent on membrane permeability leading to leakage of enzyme.

Effect of organic solvents on protease activity was monitored. The enzyme was found to be highly tolerant to all the solvents tested including xylene and toluene and was more tolerant than the organism itself.