CHAPTER 5

CONCLUSION AND FUTURE PERSPECTIVE

We have developed a two step methodology to purify protease by ammonium sulphate precipitation followed by DEAE ion exchange chromatography from *Pseudomonas thermaerum GW1*. The purified enzyme was thermostable at 60°C, pH 8.0 and had a molecular weight of 43Kda as shown by casein zymography. It retained activity in presence of organic solvents. We report isolation of protease that retained its activity in organic solvents, thus it can have high potential in industrial applications as a biocatalyst for peptide synthesis in organic media. Several reports showed that peptide synthesis could be enhanced by the addition of organic solvents in the reaction mixture [247]. Based on morphological, biochemical and nucleotides homology and phylogenetic analysis the microbe (GW1) was detected to be *Pseudomonas thermaerum*. The highest protease production (32 units/mg) occurred in basal medium supplemented with casein whereas lowest (9.7units/mg) protein in basal medium supplemented with Pigeon feathers after 48 hrs of cultivation. We report 6.08 fold purification of enzyme following ammonium sulphate precipitation and DEAE-cellulose chromatography.

The molecular weight of the enzyme was estimated to be approximately 43000 daltons as shown by casein zymography studies. In our attempt for further purification we have found traces of enzyme activity of 90 kda (data not shown) by casein zymography studies. The optimum pH for the proteolytic activity was pH 8.0 and enzyme remained stable between pH 5 -11 at 60°C. Mn²⁺ (5mM) strongly activated enzyme activity by 5 fold, while Cu²⁺, Mg²⁺ and Ca²⁺ moderately activated enzyme activity, where as Zn²⁺, Fe²⁺ and Hg²⁺ inhibited enzyme activity. The protease produced was stable in presence of 50 % (v/v) ethylacetate and acetone. Isopropanol, methanol and benzene increased protease activity by 2.7, 1.3 and 1.1 fold respectively.

Ours is the first report that shows extracellular production of proteases from *Pseudomonas thermaerum* GW1 strain, the enzyme from this source lost its activity in the presence of glycerol, sucrose and iron. It lost its activity in the presence of iron. We conclude that the
buffer best suited for *Pseudomonas thermaerum* protease kinetic studies should minimize the use of glycerol and sucrose during dialysis however for solubilizing the inhibitors, hydrophobil peptidyl substrates methanol, PEG and BSA can be used for kinetic studies since the protease is not sensitive to them.

Protease was also purified from senesced leaves of *Lantana camara*, an invasive noxious weed by ammonium sulphate precipitation and gel filtration chromatography. The enzyme showed 28.31 fold purification with a yield of 6.19%. The enzyme was shown to have a low molecular weight of 43 kda by SDS-PAGE. It was strongly activated by metal ions such as Cu$^{2+}$, Zn$^{2+}$, Mg$^{2+}$, Co$^{2+}$ and Mn$^{2+}$. It remained active at 60°C, pH 10.5 even after 1 hour of incubation when casein was used as substrate. The compatibility of the enzyme was studied with commercial and local detergents, 60% activity of the enzyme was retained even after 1 hour of incubation at pH 10.0. E-64 inhibited enzyme activity by 99.6% (at10mM concentration), this indicated that it is a cysteine alkaline protease.

We have developed a two step methodology to purify protease from senesced leaves of *Lantana camara*, an invasive noxious weed. The enzyme was thermostable at 60°C and retained activity in presence of detergents. The easy availability of the senesced leaves of this common shrub makes it a cheaper enzyme source which is thermostable and makes it a good choice to be an additive in the detergents. We report simple and economic purification of Protease from senesced leaves of *Lantana camara*, (a common shrub) this weed makes the large-scale production of protease possible, thus enabling to explore various industrial as well as Biotechnological applications.

Future studies regarding upgrading the protease production technology from laboratory to a large-scale process, allowing for a new green industrial process to be developed especially where enzymatic treatment of protein fibers, like hair wool and silk is involved so that there is significant reduction in the chemical use and cost. Development of new formulations for industrial and domestic wool carpet cleaning, garment washing, dyeing processes for protein fibers by pre-treatment with the proteases is to be performed. The amino acid sequence determination of purified alkaline protease from senesced leaves of *Lantana camara* and *Pseudomonas thermaerum* can be performed and checked for innovative application in other biotechnology industries.
It needs to be checked whether loss of activity of the native protease from *P. thermaerum* in presence of glycerol, toluene and sucrose is due to the instability or unfolding of the enzyme, slow dissociation of active dimer to inactive monomer and studies can be designed to check for the stabilization of activity with an excess of competitive inhibitor, by using fluorogenic substrates and HPLC assay method etc.

Also in future we aim that to get selective processes and clean products that are very robust to fit in the industrial environment and prevent industrial pollution and providing a greener environment. We will try to use natural resources instead of traditional chemicals while developing new methods for additives in detergents. For example the *Sapindus detergens* naturally contains saponin also known as nature’s soap. It naturally soften the clothing, and have been in use throughout India and Nepal for centuries as a versatile and gentle cleaning agent.