Summary

In the present study entitled **"Protease from *Serratia marcescens*: Production, purification and characterization"**, *Serratia marcescens* PPB-26 has been isolated from soil samples collected from various regions of Himachal Pradesh. This organism produces a very active protease, the production and reaction conditions for which were optimized. The protease from the isolate has also been purified and evaluated for several applications. Major findings and observations of the present investigation are summarized below.

6.1 Isolation and screening of protease producing bacterial isolates

Two hundred and twenty one bacterial isolates were isolated from soil samples of Himachal Pradesh by enrichment culture method. Of these, forty isolates showing protease activity, as detected by primary screening done on skimmed milk agar plates (1% and 5%), were selected for quantitative determination of proteolytic activity. Among these, PPB-26 isolate exhibited the maximum protease activity (8.0 U/mg protein). On the basis of morphological, physiological and biochemical characteristics as well as by 16S rDNA sequencing, the bacterial isolate PPB-26 was identified as *Serratia marcescens*. The 16S rDNA sequence of the isolate was submitted in NCBI and was assigned the GeneBank accession number KJ735909. The phylogentic tree prepared from this sequence also indicated its similarity with previously reported *S. marcescences* strains.

6.2 Optimization of *S. marcescens* PPB-26 protease activity

6.2.1 Optimization of cultural conditions for protease production

Of the twelve culture media screened for hyper production of protease enzyme by *S. marcescens* PPB-26, medium M2 (glucose 2%, yeast extract 0.4%, KH_2PO_4 0.15%, $MgSO_4$ 0.05%, $CaCl_2$ 0.05%, Casein 1%) emerged as the most suitable medium with 9.0 U/mg protein of protease activity observed in this medium. The effect of medium pH on protease production showed that neutral pH 7 is most suitable for the production of this enzyme by *S. marcescens* PPB-26 although the protease production was fairly stable even up till pH 9.5. The optimum protease production was seen at 30°C temperature and a decline in the protease production was observed beyond 40°C. Among the carbon and nitrogen sources the highest production of

protease was observed with 2.5% glucose (9.2 U/mg protein) and 1.5% tryptone (9.5 U/mg protein) respectively. The inoculum size did not have much effect on the protease production since beyond 2% the relative increase in protease activity as compared to increase in the inoculum size was not high. The inoculum size of 2% was thus chosen as best suited for protease production by *S. marcescens* PPB-26. In presence of various metal ions (1mM) the protease production was highest when the media was supplemented with $CaCl_2$, as the protease activity was increased to 10.0 U/mg protein. Other metal ions had no effect on the protease production except $MnCl_2$ which showed an inhibitory effect (4.0 U/mg protein). The activity profile of *S. marcescens* PPB-26 protease showed that its production is related to cell growth and maximum activity is observed after 24 h incubation at 30°C towards the end of the exponential and the beginning of stationary phase of the organism. Response surface methodology was also applied to further optimize the culture conditions and it resulted in a 1.75 fold (75%) increase in the protease activity to 17.6 U/mg protein with yeast extract 1%, $CaCl_2$ 0.25% and KH_2PO_4 0.5% being the optimized factors.

6.2.2 Optimization of reaction conditions

Reaction conditions for the assay of protease activity of *S. marcescens* PPB-26 were optimized. The protease activity was increased by 1.14 fold to 20.0 U/mg protein under the optimized reaction conditions i.e. Tris-HCl buffer 0.15M, pH 7.5; temperature 30°C; substrate concentration 0.8% and reaction time 10 min. The protease of *S. marcescens* PPB-26 had K_m = 0.3% and V_{max} = 34.5 μmoles mg^{-1} min^{-1} protein. It enzyme was also found to be fairly stable with most metal ions except Co^{2+} and Zn^{2+}. An increase in the activity was however observed with Fe^{2+} and Cu^{2+} ions. The protease of *S. marcescens* PPB-26 could also tolerate the NaCl concentration upto 0.8M. The protease was found to be fairly stable in most organic solvents (50% v/v) kept at 4°C for 24 h, except isopropylalcohol in which the activity was severely affected. The maximum stability of the enzyme was however observed with methanol and ethanol.

6.3 Purification and characterization of *S. marcescens* PPB-26 protease

Protease of *S. marcescens* PPB-26 was best precipitated with ethanol when compared to acetone and ammonium sulphate fractionation. The organic solvent fractionation was carried out with 40% (v/v) ethanol saturation which was a very

specific cut and it resulted in removal of most of the contaminating proteins. Protein precipitation with ethanol resulted in 2.25 fold purification of the crude protein and gave a 28% yield. In the SDS PAGE analysis, these precipitates revealed only two bands. Further purification was thus simplified and carried out by a single step process using DEAE-cellulose ion exchange chromatography. It resulted in 3.8 fold purification of the protein precipitates and gave a yield of 20% and a specific activity of 76.2 U/mg protein. SDS PAGE and native PAGE analysis of the purified *S. marcescens* PPB-26 protease revealed that the protease was a monomer of 50 kDa size. The MALDI TOF analysis of the purified protease showed that its sequence (39%) has maximum similarity with earlier reported metalloprotease of *S. marcescens*. The N-terminal sequence of the purified protein also revealed homology equivalent to 100% with the N-terminal sequences of earlier characterized metalloproteases of *S. marcescens* (gi 157833779, gi 3114529, gi 1431808, gi 418205677, gi 167487383, gi 672090854, gi 157833854, gi 218198433, gi 47591).

The reaction conditions for optimal activity of the purified protease of *S. marcescens* PPB-26 were studied. The purified protease was found to show best activity under the following reaction conditions: Tris-HCl buffer 0.15M, pH 7.5; 30°C incubation temperature; 0.25% casein substrate and 10 min incubation time. Reaction optimization improved the activity of the enzyme by one fold and the resultant specific activity of the purified protease was 76.5 U/mg protein. The purified protease of *S. marcescens* PPB-26 had K_m = 0.28% and V_{max} = 111.11 μmoles mg^{-1} min^{-1} protein. It was fairly stable in a short temperature range of 30°C-40°C for 6h. Beyond 40°C the protease lost activity quickly within the first hour of incubation. At 4°C however it showed a half life of 10 days. The activity of *S. marcescens* PPB-26 purified protease was found to be stable in the presence of most metal ions except $ZnSO_4$ and $FeCl_2$, whereas with Co^{2+}, Cu^{2+}, Mg^{2+}, Ca^{2+} and $MnSo_4$ the activity was increased. The stability of the purified protease in various organic solvents (1:1) was also tested at 4°C for 24 h. It was found to be stable in most organic solvents except isopropyl alcohol. The effect of various denaturating agents and inhibitors on the purified protease of *S. marcescens* PPB-26 was also studied. The enzyme retained activity in the presence of urea (0.5M) and PMSF (5mM) while the activity was severely inhibited with DTT (5 mM), EDTA (5 mM) and mercaptoethanol (5 mM) when incubated with these for 10 min at room temperature. Since PMSF is an

inhibitor of serine and cysteine proteases and EDTA is a specific inhibitor of metalloprotease, the severe decline in activity of the purified protease of *S. marcescens* PPB-26 when incubated with EDTA reaffirmed the MALDI TOF and N-terminal sequence analysis results which indicated that the purified protease has the maximum similarity with the metalloprotease of *S. marcescens.*

6.4 Applications of protease of *S. marcescens* PPB-26

The potential of the purified protease of *S. marcescens* PPB-26 for application in the detergent industry was checked. Firstly the stability of the purified protease in various detergents (5% v/v of 10mg/ml) was checked. It was found to be most stable with Ariel detergent followed by Tide detergent while with the non-ionic detergents (Tween-20, Tween-80, Triton X-100) the activity decreased. Secondly the activity in the presence of varying concentrations (5-30%) of oxidizing agent (H_2O_2) and bleaching agent (sodium hypochlorite) which are the usual components of commercial detergents was checked. The protease activity decreased with the increase in their concentrations becoming zero above 20%. The effect of the purified protease on protein (blood) stain removal from cloth was then checked. The purified protease (10 U/ml) was found to be more effective in stain removal than Ariel (5% v/v of 10mg/ml) but the efficiency of stain removal was increased when the two worked together. The application of purified protease of *S. marcescens* PPB-26 in silver recovery, by removing the gelatin from the used X-ray films, was also investigated. The protease (10.0 U/ml) was found to be capable of removing gelatin from the film (1 cm x 1 cm) completely in 10 min at 30°C. This was easily visualized by the discoloration of the X-ray film when the purified protease is added to it.

The potential of protease of *S. marcescens* PPB-26 as an anticoagulant was also checked. The enzyme (10.0 U/ml) was able to liquefy 4ml of coagulated blood in under a minute. The purified protease was also found to have anti-microbial activity against *B. cereus* (Gram positive), *S. aureus* (Gram positive) and *S. dysenteriae* (Gram negative). However towards gram-positive *L. monocytogenes* and gram-negative *E. coli*, no such anti-microbial activity was shown. The protease of *S. marcescens* PPB-26 thus also has the potential to act as a biocontrol agent.