Discussion

Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes. They are ubiquitous in all living beings and show a vast diversity of physiochemical and catalytic properties. The present study was focused on isolation of protease producing bacteria from the soil samples collected from various regions of Himachal Pradesh. Growth conditions of the bacteria were then optimized to achieve maximum protease activity. The enzyme was further purified and its application in various industrial processes was tested. The outcomes of the experiments are being discussed in this chapter.

5.1 Isolation and screening of protease producing bacterial isolates

Two hundred and twenty one bacterial strains were isolated from soil using enrichment culture method in minimal salt medium. Soil has served as a source of numerous protease producing bacterial isolates such as *Comomonas kerstersii* KSM7 which was isolated using enrichment medium having the similar composition (Swamy *et al.,* 2014), *Bacillus megaterium* (Asker *et al.*, 2013); *B. subtilis* (Padmapriya and Williams, 2012) and *Pseudomonas thermaerum* (Gaur *et al.*, 2010). These isolates were subjected to primary screening for protease activity on skimmed milk agar plates. Skimmed milk agar is used for the demonstration of proteolysis of casein (Frazier and Rupp, 1928). The ability to hydrolyze casein in milk is determined by formation clear zones of digestion around the bacterial colonies and is a widely used screening method for proteases (Chang *et al.*, 2013). Forty isolates were screened by this process and subjected to quantitative determination of protease activity (Manachini *et al.*, 1988). Of all isolates PPB-26 exhibited the maximum protease activity (8.0 U/mg protein) and was identified as *Serratia marcescens* by 16S rDNA sequencing done at MTCC, IMTECH Chandigarh, and by biochemical and physiological analysis (Bergey's Manual). An activity of 0.66U/mg protein (Sayali *et al.*, 2013) and 0.782Â±0.041U/mg protein (Krishna *et al.*, 2011) has been previously reported from proteases of *B. subtilis*. In another report proteases produced by food borne bacterial strains showed the following specific activities respectively: *B. cereus*: 1.851 U/mg protein; *P. vulgaris*: 1.904 U/mg protein; *P. mirabilis*: 2.126 U/mg protein and *E. aerogenes:* 1.692 U/mg protein (Prakash *et al.*, 2011).

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

5.2.1 Optimization of culture conditions for protease production

S. marcescens PPB-26 showed highest protease activity in M2 medium (glucose 2%, yeast extract 0.4%, KH_2PO_4 0.15%, $MgSO_4$ 0.05%, $CaCl_2$ 0.05%, Casein 1%) and least in M4 (soya meal 1.5%, sucrose 0.5%, starch 5%, $MgCl_2$ 0.05%, Na_2CO_3 1%). Increasing levels of soyabean meal have been reported to cause a linear inhibitory effect on the protease activity (Chong *et al.*, 2002). Anti-trypsin inhibitors present in the soyabean have been proven to reduce the capability to digest the proteins (Olli *et al.*, 1994; Haard *et al.*, 1996). The optimum media pH for protease production in *S. marcescens* PPB-26 was found to be pH 7 though the enzyme was secreted in a broad range from pH 5.5 to 9.5. At a higher pH metabolic action of the bacterium could have been suppressed thus decreasing the enzyme production. Similar trends have been observed in protease by *Bacillus* sp. (Prakasham *et al.*, 2006; Okafor and Anosike, 2012). A metalloprotease secreted by *Salinivibrio* sp. strain AF-2004 (Amoozegar *et al.*, 2007) and a de-hairing protease by *B. cereus* strain AT (Vijayaraghavan *et al.*, 2014) have also been reported to exhibit broad pH ranges (5.0–10.0 and 6.0-9.0 respectively). *S. marcescens* PPB-26 also showed a sharp increase in protease production as the incubation temperature was increased from 25°C to 30°C. Beyond 30°C there was a steady decline in protease production with the enzyme activity remaining fairly stable till 40°C. Similar temperature ranges for production of proteases have been previously reported by *B. pumillus* ATCC7061 (Gomaa, 2013) and also by *B. cereus* strain CA15 (Uyar *et al.*, 2011).

Neutral and alkaline proteases hold great potential for application in detergent and leather tanning industries due to increasing trends in developing environment friendly technologies (Rao *et al.*, 1998). The neutral metalloproteases, due to specificity for hydrophobic amino acids, generate less bitterness in hydrolyzed food proteins and hence are valuable in food industries, low thermostability being an advantage for controlling their activity (Barrett, 1995; Rao *et al.*, 1998).

Of the different carbon sources 2.5% glucose was the most preferred carbon source for protease production by *S. marcescens* PPB-26 whereas sucrose, galactose and fructose were least preferred. Similar preference towards glucose was reported by Gomma *et al.*, 1990, Sen and Satyanarayana, 1993, and Pastor *et al.*, 2001. The

protease yield decreased with increase in concentration of glucose in the production medium. This observation is consistent with the effect of glucose on protease production by previously reported strains (Razak *et al.*, 1994; Purva *et al.*, 1998). Glucose has previously been reported as the suitable carbon source for protease production by *Bacillus* sp. (Fujiwara and Yamamoto, 1987), *S. marcescens* (Mohankumar, 2009) and *Bacillus* sp. B18 (Fujiwara *et al.*, 1993) which utilized 1% glucose and by *B. licheniformis* N3 (Sinha and Satyanarayana, 1991) which utilized 2% glucose for optimal protease production.

Organic nitrogen sources were found to be better sources for protease production in *S. marcescens* PPB-26 than the inorganic nitrogen sources which resulted in decreased protease activity. A similar trend was reported in case of *B. pumilus* protease (Feng *et al.*, 2001) and serratiopeptidase enzyme of *S. marcescens* (Mohankumar, 2009). More specifically 1.5% tryptone showed the best results with protease activity decreasing gradually with increase in tryptone concentration till 3.5%, beyond which a sharp decline in activity was recorded. Of the different metal ions studied for effect on protease production most showed no effect on enzyme activity except managanese chloride which severely reduced the enzyme activity. An increase in protease activity was however observed with calcium chloride. The metal ions stabilize and enhance the production and activity of protease enzyme (Janssen *et al.*, 1994). A similar enhancement of production of *Pseudomonas aeruginosa* protease IV by calcium has been reported earlier (Marquart *et al.*, 2005).

The growth kinetic studies of *S. marcescens* PPB-26 revealed that the highest values of protease activity were achieved at the end of exponential growth phase or during stationary phase, which indicates that *S. marcescens* PPB-26 protease is a metabolite whose production is associated with cell growth. Many of the *Bacillus* sp. have been reported to produce extracellular protease during the post-exponential and stationary phases of growth (Schaeffer, 1969; Ward, 1983). Extracellular protease of another *S. marcescens* strain (Ustáriz *et al.*, 2008) has also been reported to show a similar behavior.

5.2.2 Factorial design of experiments for enhancing protease production

Application of response surface methodology for improving production of metalloprotease from *S. marcescens* has not been reported before. Plackett-Burman

design of the experiments revealed that yeast extract, KH_2PO_4 and $CaCl_2$ were the major factors which affect protease enzyme production by *S. marcescens* PPB-26. Central composite design was used for optimization of values of positive factors in the media for maximum protease production. After performing factorial design, 1.75 fold (75%) increase in protease activity to 17.5 U/mg protein was recorded due to the interaction of various variables. In *Pseudomonas aeruginosa* MCM B327, 1.3 fold increase in protease activity has been reported (Zambare *et al.*, 2013) after application of the response surface methodology.

5.2.3 Optimization of reaction conditions

S. marcescens PPB-26 protease showed maximum activity at pH 7.5 of Tris-HCl buffer (0.15 M). The ionic strength of buffer did not have much effect on the protease activity. The optimum temperature for maximum hydrolysis of casein by protease of *S. marcescens* PPB-26 was found to be 30°C. At higher temperatures beyond 40°C the enzyme activity decreases indicating that the enzyme is not stable at higher temperatures. The protease activity faced product inhibition at substrate concentrations beyond 0.8% due to accumulation of product in the reaction. 0.8% casein was thus the optimal substrate concentration for activity of *S. marcescens* PPB-26 protease. The optimal reaction time was found to be 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. Co^{2+} and Zn^{2+} had negative effect on the activity whereas with Fe^{2+} a positive effect on the enzyme activity was observed. Similar increase in enzyme activity in the presence of Fe^{2+} ions has been reported earlier (Yang *et al.*, 2000). Also the negative effects of Co^{2+}, Zn^{2+} and Mn^{2+} have previously been reported on the production of protease from *B. stearothermophilus* (Rahman *et al.*, 1994) and *B. polymyxa* (Kaur *et al.*, 1998). The catalytic nature of enzyme (whether to promote or inhibit enzyme activity) is determined by an ion's ability to occupy a metal-binding site at the active centre (Coolbear *et al.*, 1992).

The protease enzyme of *S. marcescens* PPB-26 showed tolerance to high concentrations of NaCl (0.8M). There was a decrease in activity of protease as the NaCl concentration increased. This might be due to precipitations of substrate at high salinities. The protease of *Bacillus* sp. NG-27 (Sumandeep *et al.*, 1999) and other alkaline proteases (Kembhavi *et al.*, 1993; Manachini and Fortina, 1998) have also been reported to exhibit high salt tolerance.

Proteases are usually inactive or show a low activity in non-aqueous media (Gupta, 1992; Vulfson *et al.*, 2001). Therefore finding solvent stable proteases has made an extensive area of research (Geok *et al.*, 2003). Several low-water stable proteases have been identified in *Pseudomonas* sp (Ogino *et al.*, 1995; Geok *et al.*, 2003). Thus the effect of various organic solvents on the protease of *S. marcescens* PPB-26 was tested over a 24 h time period. The enzyme showed considerable stability in most organic solvents except in isopropyl alcohol where the activity was greatly inhibited. Similar observations on the effect of organic solvents on protease activity have been made earlier (Ogino *et al.*, 1995; Yang *et al.*, 2000). Very few reports are available in literature concerning the screening of microorganisms which produce *organic solvent-stable proteases* (Gupta and Khare, 2006). However the enzyme activity in organic solvents is, in general, lower than that in an aqueous environment (Klibanov, 1997). The reasons for this low activity are limited diffusion of enzymes, partial denaturation of enzymes in lyophilization and reduced flexibility of proteins in anhydrous solvents.

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

The extracellular protease enzyme of *S. marcescens* PPB-26 was purified in a single step process. The enzyme being fairly stable, most of the purification steps were performed at room temperature. Very few metalloproteases from *Serratia* sp. have been purified and studied in detail. For precipitation of the protease enzyme, ethanol fractionation showed the best results in comparison to acetone and ammonium sulphate fractionation. Since the cut was highly specific (30% - 40% ethanol saturation) most of the contaminants got removed during this step hence the low yield (28%) and 2.25 fold purification. This was later confirmed in the SDS-PAGE analysis of the protein precipitates obtained by ethanol fractionation which revealed only two bands. Similar results have been reported for alkaline serine protease of *B. clausii* GMBE 22 (Kazan *et al.*, 2009), *Bacillus* sp. APR-4 protease (Kumar and Bhalla, 2004) and *Chromohalobacter* sp. TVSP101 protease (Vidyasagar *et al.*, 2009). One of the practical advantages of organic solvent fractionation over ammonium sulphate fractionation is that owing to the change in the density of the solution caused by addition of organic solvent, the protein precipitates settle down more readily (Wilson, 1997). Solvent precipitation thus yields a product of higher purity and activity than salt precipitation (Moon and Parulekar, 1991). The precipitates obtained by organic

solvent fractionation were dissolved in 0.15M Tris-HCl buffer pH 9.0. The protease from these precipitates was eluted at 0.6M NaCl from a DEAE-cellulose matrix suggesting that the enzyme possessed high negative charge over its surface at pH 9.0. Due to elution of the required protein at a high NaCl concentration most of the contaminating proteins bound in the matrix got eluted in fractions of lower salt concentrations leaving only the required protease to be eluted towards the end. The protease was thus purified using ion exchange chromatography to 3.81 fold with 20% yield and the specific activity of 76.2 U/mg protein was achieved. In *S. rubidaea* a fold purification of 1.5 of protease has been reported (Doddapaneni *et al.*, 2007), while 1.49 fold purification with 74.66 U/mg protease activity has been reported in *B. subtilis* protease (Ahmed *et al.*, 2011). The SDS and native PAGE analysis of the purified fraction showed the enzyme to be a monomer of 50 kDa. Purified metalloproteases of similar sizes (58kDa, 52kDa) have been previously reported from *S. marcescens* AP3801 (Morita *et al.*, 1997) and *Serratia* sp. KCK (Kim *et al.*, 2007). MALDI-TOF and N-terminal sequence analysis of the purified protein revealed that the sequences of the purified protein showed maximum homology with earlier reported metalloproteases of *S. marcescens* thus reaffirming that the purified protein was a *S. marcescens* metalloprotease.

Purified protease of *S. marcescens* PPB-26 exhibited maximum activity at pH 7.5 of Tris-HCl buffer (0.15M). Similar trend has been reported in the *S. marcescens* S3-R1 protease (Nam *et al.*, 2013). Neutral and alkaline proteases hold great potential in detergent and leather tanning industries due to the increasing trend in developing environment-friendly technologies (Rao *et al.*, 1998). The purified protease was more active at 30 °C and a decrease in activity was recorded beyond 40 °C. A similar trend was seen in *S. marcescens* S3-R1 protease (Nam *et al.*, 2013) and in *S. rubidaea* protease (Kumar *et al.*, 2007). The protease of *S. marcescens* PPB-26 is hydrophobic in nature as observed during elution profile in DEAE chromatography. Thus an increase in temperature leads to breaking of hydrogen bonds and non polar hydrophobic interactions, which resulted in loss of enzyme activity beyond 40 °C. *S. marcescens* PPB-26 protease could optimally hydrolyze upto 0.25% of casein (substrate) and very little increase in activity was recorded above this concentration due to substrate inhibition. The protease of *S. marcescens* PPB-26 has half life of 15 days at 4°C whereas at 30 °C and 40 °C it remains stable for a period of 6 h. Co^{2+},

Cu^{2+}, Mg^{2+} and Ca^{2+} had a positive effect on purified protease activity while Zn^{2+} showed an inhibiting effect on it. The protease was most stable in Ca^{2+} (2mM). Earlier investigations had shown that Ca^{2+} has a significant stabilizing effect on the purified protease (Kumar and Bhalla, 2004) and in some cases it also stimulated the activity of protease (Manachini *et al.*, 1988). Increase in purified protease activity with Mg^{2+} has also been reported (Shimogagi *et al.*, 1991). *V*max for the purified protease was calculated as 111 U/mg protein and its *K*m was 0.28%.l

The purified protease of *S. marcescens* PPB-26 was stable in most organic solvents except in isopropyl alcohol which inhibited the enzyme activity. A slight increase in the activity was observed with methanol and ethanol which is similar to the results obtained with the protease of *Bacillus* sp. APR-4 (Kumar and Bhalla, 2004) and *Selenomonas ruminantium* protease (Ravindran *et al.*, 2012). With denaturating agents and inhibitors the purified protease was found to be stable with urea and PMSF while the enzyme activity was severely decreased with mercaptoethanol, DTT and EDTA. The inhibitory effect of EDTA confirms that the protease of *S. marcescens* PPB-26 is a metalloprotease and requires metal ions for its functioning (Yang *et al.*, 2000; Kumar and Bhalla, 2004).

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

The largest application of protease enzyme is in the detergent industry. Many commercial detergents contain proteolytic enzymes majority of which are produced by the members of genus *Bacillus* (Samal *et al.*, 1989). Protease from other sources thus holds high potential in providing an alternate source of protease with novel properties. At times certain cleansing formulations are supplemented with oxidizing agents to enhance the brightness of or to sterilize the washed material (Greene *et al.*, 1996, Manachini and Fortina, 1998). Besides these, due to environmental and energy constrains the protease should preferably be active at normal temperatures and normal pH levels. Taking the above points into consideration investigations were carried out for testing the potential of protease of *S. marcescens* PPB-26 as a component of detergents. The enzyme was fairly stable in the detergents while it showed an increased activity with Ariel. The effect of oxidizing agent and bleach resulted in decreased activity of the enzyme reaching almost zero beyond 15%v/v concentration. When applied for blood stain removal from a cloth, the enzyme very effectively removed the blood stain within 5 min. Whereas in the presence of the Ariel detergent

the enzyme showed higher efficiency and the stain removal was done within 2 min. Greene *et al.*, (1996) had reported that enzyme supplemented detergent was considerably more effective at removing the soiling agents (blood, milk and carbon black) than the control. The use of enzyme as cleansing agents is expected to reduce the amount of detergent and energy required for cleaning, as enzymes generally require only mild conditions for action.

Alkaline proteases from neutrophilic *B. subtilis* are the most commonly used enzymes in silver recovery. Using enzymes for this industrial process instead of chemicals renders this as a more eco-friendly process having lesser energy requirement as well. The protease of *S. marcescens* PPB-26 (10 U/ml) was able to completely hydrolyze gelatin on X-ray film (1 cm x 1 cm) in 10 min thus indicating its immense potential in recovery of silver from the used X-ray films.

The application of protease of *S. marcescens* PPB-26 as an anti-coagulant in the pharmaceutical industry was also studied. The purified protease (10 U/ml) was able to liquefy the colagulated blood (4 ml) in 1 min which was lesser than the time taken (30 min) by the protease of *Virgibacillus dokdonensis* VIT P14 (Devi *et al.*, 2012). The ability of protease to digest different natural substrates including blood clot suggests the usefulness of this enzyme for different applications such as waste treatment, in removing the blood proteins from the medical instruments (Kumar *et al.*, 1998) and other related applications.

Proteases are not merely restricted to digestive purposes and remodeling of extracellular matrix and tissues but are also key factors for the induction of physiological immune responses. The proteases have been shown to be important in plant defense against biotic stresses (Guevara *et al.*, 2004). For example, a cysteine endoprotease confers resistance to maize against fall armyworm (Jiang *et al.*, 1995). Exopeptidases, such as leucine aminopeptidase-A and tomato wound-induced carboxypeptidases have been suggested to play important roles in plant defence (Chao *et al.*, 1999) by inactivating proteins essential for pathogen or insect growth and pathogen spread. Rodrigo *et al.*, (1989) reported the constitutive expression of an aspartic protease that degrades pathogenesis-related proteins (PR proteins) in the intercellular fluid of tobacco and tomato plants. Thus the presence of antimicrobial activity in the purified protease of *S. marcescens* PPB-26 was studied by well diffusion method. The antibacterial property of the enzyme (10U/mg) was tested

against *E. coli, S. aureus, B. cereus, L. monocytogenes* and *S. dysenteriae.* The enzyme showed zones of inhibition with *S. aureus, B. cereus* and *S. dysenteriae* whereas no effect was seen on gram-positive *L. monocytogenes* and gram-negative *E. coli.*

Gram positive *S. aureus*, though not always pathogenic, are a common cause of skin infections (e.g. boils), respiratory disease (e.g. sinusitis), and food poisoning. Disease-associated strains often promote infections by producing potent protein toxins and expressing cell-surface proteins that bind and inactivate antibodies. *B. cereus* is a gram positive endospore forming bacteria responsible for a minority of foodborne illnesses (2–5%), causing severe nausea, vomiting and diarrhea. *Bacillus* food borne illnesses occur due to survival of the bacterial endospores when food is improperly cooked. *S. dysenteriae* are gram-negative, nonspore-forming bacteria that spread by contaminated water and food and cause the most severe dysentery because of their potent and deadly shiga toxin. To reduce the presence of these pathogens, alternative interventions have been studied. However, increasing consumer's demand for natural ingredients has made the investigations of effectiveness of natural antimicrobials necessary. Hence in this context the protease of *S. marcescens* PPB-26 possesses immence potential as a natural biocontrol agent.