Materials and Methods

3.1 Isolation of protease producing microorganisms from various regions of Himachal Pradesh

3.1.1 Sample collection

The soil samples were collected from various regions of Himachal Pradesh (Shimla, Kullu, Manikarn, Manali, Kinnaur and Bilaspur), from sites that were rich in decaying garden waste, farm waste and industrial effluents. The samples were collected and brought to the laboratory in sterile sample-vials and polythene bags and kept at 4 °C till further processing.

3.1.2 Enrichment of protease producing bacteria from the soil samples

3.1.2.1 First enrichment

To 50 ml of autoclaved medium, containing: glucose 1 g ; peptone 10 g; yeast extract 0.2 g ; $CaCl_2$ 0.1 g ; K_2HPO_4 0.5 g ; $MgSO_4$ 0.1 g and casein 10 g, (pH 7.0) in L^{-1} of distilled water, 500 mg of the soil sample was added. For isolation of protease producing bacteria, enrichment was carried out at 30 °C for 24 h.

3.1.2.2 Second and third enrichment

To 50 ml of the isolation medium, 1 ml of culture broth from first enrichment was added and incubated at 30 °C for 24 h. Subsequently, 1 ml of culture broth from the second enrichment was used as inoculum for the third enrichment and incubated at 30 °C for 24 h.

3.1.3 Isolation of bacteria

After the third enrichment, 1 ml of culture was serially diluted to 10^{-4}-10^{-6} times with physiological saline. The diluted inoculum (0.1 ml) was then plated on nutrient agar plates and incubated for 48 h at 30 °C. Pure line cultures were obtained by streaking single bacterial colonies on the nutrient agar plates. These were then maintained on nutrient agar slants and stored in refrigerator at 4 °C. The isolates were sub-cultured once every month to maintain pure lines.

3.2 Screening of bacterial isolates for protease production

3.2.1 Quantitative determination of proteolytic activity

The bacterial isolates obtained by enrichment culture were streaked on skim milk agar plates (5% skim milk). Skim milk agar is used for the demonstration of proteolysis of casein (Frazier and Ripp, 1928). The ability of the isolates to hydrolyze casein in the milk was determined by noting the clear zones of digestion formed around the bacterial colonies after incubating the plates at 30 °C for 24 h.

3.2.2 Qualitative determination of proteolytic activity

3.2.2.1 Preparation of seed culture

The seed culture was prepared in nutrient broth. The screened colonies were inoculated in 50 ml of seed medium and incubated at 30 °C for 24 h in an incubator shaker.

3.2.2.2 Production of protease

To 50 ml of minimal medium (0.1% glucose; 1% peptone; 0.02% yeast extract; 0.01% $CaCl_2$; 0.05% K_2HPO_4; 0.01% $MgSO_4$; 1% casein; pH 7) in 250 ml Erlenmeyer flask, 1ml of the 24 h seed culture was added. This was then incubated at 30 °C for 24 h in an incubator shaker (155 rpm), and centrifuged at 5000 x g for 30 min at 4 °C and the supernatant was assayed for the extracellular protease activity.

3.2.2.3 Protease assay (Manachini *et al.*, 1988)

Reagents:

i) Casein substrate: 0.5% (w/v) of casein dissolved in 50 mM potassium phosphate buffer (pH 7.0)

ii) Stopping reagent: 5.0% (w/v) of Trichloroacetic acid (TCA) in distilled water

Procedure:

To 4 ml of substrate solution, 1 ml of enzyme (supernatant from 3.2.2.2) was added and incubated for 20 min at 30 °C. The reaction was stopped with 5 ml of the stopping reagent and vortexed. The reaction mixture was centrifuged at 10,000 x g for 5.0 min and the optical density (OD) was taken at 275 nm (UV-160 A Shimadzu Spectrophotometer).

3.2.2.4 Standard curve

The standard curve was prepared with tyrosine (10-100 μg/ml). One unit of the enzyme activity is defined as the amount of enzyme required to release one μg of tyrosine/ml under assay conditions.

3.2.2.5 Protein quantification (Bradford, 1976)

The protein concentration in samples was estimated by Bradford method (1976) using BSA as a standard.

Reagents:

A. Bradford stock solution

Ethanol (95%)	100 ml
Phosphoric acid (88%)	200 ml
Commassie blue G-250	350 mg

B. Bradford working solution

Distilled water	425 ml
Ethanol (95%)	15 ml
Phosphoric acid (88%)	30 ml
Bradford stock solution	30 ml

The working solution was filtered through Whatman No. 1 paper and stored at room temperature in brown glass bottle.

Procedure:

To 0.1 ml of supernatant (diluted in distilled water whenever required) 1 ml of Bradford working reagent was added and mixed immediately by vortexing. The absorbance of the reaction mixture was read at 595 nm after 2-5 min. Standard curve of protein was prepared using 20-200 μg/ml of bovine serum albumin.

3.3 Morphological and biochemical characterization of isolate PPB-26

The isolate PPB-26 which exhibited the highest protease activity out of the 221 isolates was selected for subsequent experiments. The morphological and biochemical characteristics of the isolate were studied according to the Bergey's

Manual, and it was identified at the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh.

3.3.1 Biochemical tests

The biochemical tests (indole test, methyl red test, Voges-Proskauer test, citrate test and carbon utilization tests) for the identification of bacterial isolate PPB-26 were performed according to the Bergey's Manual and by using the HiIMViCTM Biochemical Test Kit (Code No. : KB001) (HiMedia Laboratories Pvt. Ltd).

3.4 Optimization of culture conditions for protease production by *S. marcescens* PPB-26

3.4.1 Screening of media for protease production

A total of twelve different media (pH 7) were screened for the production of protease by *S. marcescens* PPB-26, keeping the incubation temperature at 30°C and the incubation time of 24 h. The composition of the media used is given below:

i) GYP medium

Glucose	1.0%
Yeast extract	0.5%
Peptone	0.5%
NaCl	0.5%
$MgSO_4$	0.025%
$CaCl_2$	0.05%

ii) GYC medium

Glucose	2.0%
Yeast extract	0.4%
KH_2PO_4	0.15%
$MgSO_4$	0.05%
$CaCl_2$	0.05%
Casein	1.0%

iii) **Starch soyameal medium (Sinha and Satyanarayan, 1991)**

Starch	2.0%
Soya meal	1.0%
$CaCO_3$	0.3%

iv) **Soyabean sucrose starch medium (Chu *et al.*, 1992)**

Soya meal	1.5%
Sucrose	0.5%
Starch	5.0%
$MgCl_2$	0.05%
Na_2CO_3	1.0%

v) **Nutrient casein broth**

Peptone	0.5%
Beef extract	0.3%
Casein	1.0%

vi) **Maltose yeast extract medium (Tsuchiya *et al.*, 1991)**

Maltose	1.0%
Yeast extract	0.4%
K_2HPO_4	0.05%
NaCl	0.2%
Na_2CO_3	1.5%
Peptone	0.5%

vii) **Seed medium (Tsujibo *et al.*, 1990)**

Glucose	1.0%
Yeast extract	0.5%
Casein	0.5%
K_2HPO_4	0.1%
$MgSO_4.7H_2O$	0.02%
Na_2CO_3	1.0%

viii) **Tryptone dextrose yeast medium (Matta *et al.*, 1997)**

Tryptone	1.0%
Dextrose	0.1%
Yeast Extract	0.5%

ix) **GYP medium (Purva *et al.*, 1998 modified)**

Glucose	1.0%
Yeast extract	0.5%
Peptone	0.5%
$CaCl_2.2H_2O$	0.02%
$MgSO_4.7H_2O$	0.02%
KH_2PO_4	0.1%

x) **Casein beef extract peptone medium (Tsuchida *et al.*, 1996)**

Casein	1.0%
Beef Extract	1.0%
Peptone	1.0%
$NaHCO_3$	1.0%

xi) **Production medium (Kobayashi *et al.*, 1995)**

Glucose	2.0%
Beef extract	1.0%
Soya meal	1.0%
KH_2PO_4	0.1%
Na_2CO_3	1.0%

xii) **Minimal media**

Glucose	0.1%
Peptone	1.0%
Yeast extract	0.02%
$CaCl_2$	0.01%
K_2HPO_4	0.05%
$MgSO_4$	0.01%
Casein	1.0%

Of these media, the one in which isolate PPB-26 showed the highest protease activity was selected for subsequent studies.

3.4.2 Effect of pH

The effect of pH on extracellular protease production by *S. marcesence* PPB-26 was investigated by culturing it at 30 °C on the optimized medium, at different pH (4.0 -10.5) for 24 h and the protease activity in the culture supernantant was assayed.

3.4.3 Effect of temperature

The effect of temperature on the protease production by *S. marcesence* PPB-26 was studied by growing the organism in optimal medium (pH- 7), at different temperatures (25-60 °C) for 24 h and then assaying the culture supernantant.

3.4.4 Effect of carbon sources

The enzyme production was studied in the presence of various carbon sources (2%) viz., starch, sucrose, maltose, mannitol, glycerol, lactose, galactose, fructose, dextrose, sodium acetate, sodium citrate and sodium succinate for 24 h at 30°C. The effect of different concentrations (1-4%) of the selected carbon source on the protease production by *S. marcesence* PPB-26 was also studied.

3.4.5 Effect of nitrogen sources

The protease production by *S. marcesence* PPB-26 was monitored in the presence of various organic (1%) viz., casein, yeast extract, beef extract, peptone, soyabean meal, tryptone and inorganic (1%) viz., urea, $(NH_4)_2SO_4$, $(NH_4)2PO_4$, $NaNO_3$ nitrogen sources for 24 h at 30°C. The effect of different concentrations (1-4%) of the selected organic nitrogen source was also studied.

3.4.6 Effect of inoculum size

To the production medium different volumes (1-10%) of 24 h old preculture of *S. marcesence* PPB-26 was added. After 24 h incubation at 30°C, the protease activity in the culture supernatant was assayed.

3.4.7 Effect of metal ions

To study the effect of metal ions on protease production, the production medium was supplemented with 1mM concentration of $CaCl_2.2H_2O$, $CoCl_2.6H_2O$, $MgCl_2.6H_2O$, $MnCl_2.6H_2O$, $CuSO_4.5H_2O$, $MnSO_4.H_2O$, $FeSO_4.7H_2O$ and $ZnSO_4.7H_2O$.

3.4.8 Time course of protease production by *S. marcesence* PPB-26

To study the effect of incubation time on protease production by *S. marcescens* PPB-26, 1ml of 24 h old pre-culture was added to the production medium (50ml) and incubated at 30°C. The flask was taken out at regular intervals and the protease activity as well as the cell growth was determined, up until 56 h of incubation.

3.5 Factorial design for the enhancement of protease activity of *S. marcescens* PPB-26

3.5.1 Screening of important nutrient components using Plackett-Burman design

Plackett Burman design was used to screen the factors having positive effect on protease production by *S. marcescens* PPB-26. The medium components and physical parameters were screened for eleven variables at two levels, minimum and maximum. The eleven variables considered in the present studies include pH, temperature, dextrose, tryptone, casein, yeast extract, $ZnSO_4$, KH_2PO_4, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$ and NaCl. These variables were investigated and 12 experiments were carried out. Design Expert (Version 9.0) was used for Plackett-Burman design and regression analysis. The effect of each variable was calculated using the following equation:

$$E= (\Sigma M_+ - \Sigma M_-)/N$$

Where E is the effect of the tested variable, and M_+ and M_- are responses (enzyme activity) of trials at which the parameter was at its higher and lower level respectively, and N is the number of experiment carried out.

Table 3.1 Different media components and culture condition factors screened by Plackett-Burman design for protease production by *S. marcescens* PPB-26

Factor	Low	High
Ph	5.0	9.0
Temperature	20	40
Dextrose	0.5%	5.0%
Tryptone	0.1%	2.0%
Casein	0	2.0%
Yeast extract	0.1%	1.0%
$ZnSO_4$	0	0.01%
KH_2PO_4	0	0.5%
NaCl	0	1.0%