Isolation and characterization of psychrotrophic proteolytic bacteria from landfill site under temperate climatic conditions of Kashmir Himalaya

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The temperate climatic regions face the problem of waste accumulation due to lower environmental temperatures. However, these regions harbor cold active microbes viz. psychrotrophic proteolytic bacteria that play an important role in the degradation of proteaceous materials of the waste stream. Hence in the present study psychrotrophic proteolytic bacteria were isolated from waste samples collected from landfill site by using random sampling method under environmental temperature of 10°C. By using serial dilution and spread plate technique a total of 8 morphologically different psychrotrophic proteolytic bacteria were isolated on skim milk agar media at pH of 7.0 and temperature of 10°C after 48 hours. Under in-vitro conditions all the isolates produced significant quantities of protease over the control and diameters of hydrolysis zones ranged between 2 to 18 mm at temperature range of 5 to 20°C and after 72 hours. The corresponding quantitative protease activities of the isolates was significant that ranged between 0.5 to 2.25 U/ml and the isolate PB2 was most efficient with highest protease activity of 2.25U/ml at 20°C. Based on 16SrRNA analysis the isolate was identified as Pseudomonas florescence with 96% similarity. It was concluded that the isolates can grow in wide ranges of temperature and could be used for enhanced decomposition of organic wastes during lower temperature conditions in cold regions. Further the isolates could have industrial applications due to the production of cold active proteases that would help economic benefits through energy conservation.
Isolation and Characterization of Psychrotrophic Proteolytic Bacteria from Landfill Site under Temperate Climatic Conditions of Kashmir Himalaya

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Abstract
The temperate climatic regions face the problem of waste accumulation due to lower environmental temperatures. However, these regions harbor cold active microbes viz. psychrotrophic proteolytic bacteria that play an important role in the degradation of proteaceous materials of the waste stream. Hence in the present study psychrotrophic proteolytic bacteria were isolated from waste samples collected from landfill site by using random sampling method under environmental temperature of 10°C. By using serial dilution and spread plate technique a total of 8 morphologically different psychrotrophic proteolytic bacteria were isolated on skim milk agar media at pH of 7.0 and temperature of 10°C after 48 hours. Under in-vitro conditions all the isolates produced significant quantities of protease over the control and diameters of hydrolysis zones ranged between 2 to 18 mm at temperature range of 5 to 20°C and after 72 hours. The corresponding quantitative protease activities of the isolates was significant that ranged between 0.5 to 2.25 U/ml and the isolate PB₂ was most efficient with highest protease activity of 2.25U/ml at 20°C. Based on 16SrRNA analysis the isolate was identified as Pseudomonas florescence with 96% similarity. It was concluded that the isolates can grow in wide ranges of temperature and could be used for enhanced decomposition of organic wastes during lower temperature conditions in cold regions. Further the isolates could have industrial applications due to the production of cold active proteases that would help economic benefits through energy conservation.
37 **Introduction**

The management of municipal solid waste (MSW) is a global problem and land-filling is considered as the common method of disposal of MSW. In developing countries like India, biodegradable MSW accounts for more than 60% of the total MSW generation. The presence of organic materials in the waste stream also contains protein rich kitchen waste that supports the growth of proteolytic bacteria [1, 2] and thus landfill sites are regarded as store houses of effective waste degrading bacteria. The bacterial cells consume the wastes of different types by producing various specific enzymes. In temperate climatic regions the decomposition of biodegradable waste is retarded during cold temperatures due to slowing down of microbial activities. But these regions contain some psychrotrophic protein degrading bacteria that could be used for decomposition of protenaceous materials of the waste. Hence the interest was developed to study the nature of extracellular enzymes secreted by these cold adopted bacteria.

Psychrotrophic bacteria are the bacteria that grew at lower temperatures (5°C) and had higher optimal growth temperatures [3]. The permanently cold areas do not only habit the psychrotrophic microorganisms but such microorganisms are also present in seasonally cold environments because of their ability to tolerate lower temperatures [4]. In these seasonally cold environments psychrotrophs are believed to play an important role in the biodegradation of organic matter. The bacteria that are adapted to lower temperatures have special type of proteins called cold acclimation proteins (Caps) that are involved in important metabolic function(s) at low temperature by maintaining membrane fluidity and/or by replacing cold-denatured peptides.

A protease is an enzyme that carries out the hydrolysis of peptide bonds between the amino acids occurring in a polypeptide chain forming the protein and cause their splitting into free amino acids [5]. Proteases have found applications in diverse fields such as detergent industry, leather processing, silk degumming, food and dairy, baking, pharmaceutical industries, silver recovery from x-ray films, waste management and others. This high demand of proteases has triggered the scientific community to isolate novel strains and optimize the process parameters for the production of proteases [6]. The production of bacterial proteases depends on a number factors like type of strain, medium composition, cultivation method, nutrient requirement, metal ions, pH, temperature, time of incubation and thermo stability [7]. Most commonly used methods for
the identification of potential enzyme producing isolates involves the morphological and biochemical methods, however, the technique of 16SrRNA gene sequencing is considered a novel and accurate method for identification of unknown species and up-to species level. Kashmir valley is a temperate climatic region and management of municipal solid waste becomes a major issue during cold environmental conditions due to slowing down of microbial activity. Hence the interest was developed to set the aim of isolation and characterization of psychrotrophic proteolytic bacteria that could be used for the development of efficient bacterial consortium for rapid composting of biodegradable solid waste. Further no such work has been done to the best of our knowledge and characterization of the isolates would serve as a base line data for characteristics of cold adapted protease producing microorganisms.

Materials & Methods

Collection of samples

By using random sampling method waste samples were collected during winter season from Achan landfill site that lies between 34° 7' 00" North and 74° 47' 38.08" East (Fig-1). The waste samples mixed with soil were collected aseptically and transported intact at ambient temperature in sealed polythene bags to the laboratory for analysis.

Isolation of psychrotolerant protease producing bacteria

The isolation of bacteria was carried on selective media (Skim milk agar) by serial dilution and spread plate technique under aseptic environment of laminar flow cabinet. 1g fresh weight of each sample was taken and added to 9ml of sterile water in test tubes and serially diluted in the range of (10^-1 to 10^-6 fold dilution). From each dilution 1ml of solution was taken and spread on Skim Milk Agar Plates (pH -7.0) (g/l) (skim milk powder-28, casein enzymatic hydrolysate-5, yeast extract-2.5, dextrose-1, agar -15) and were kept inverted at 10°C for 48 hours. The colonies that had formed a clear zone around were selected as protease positive isolates. Colonies were picked based on difference in morphology, size and color. The different isolates were sub cultured to achieve the pure cultures and preserved in nutrient agar slants at 4°C.

Screening of isolated bacteria through qualitative protease test

The pure cultures of the isolates were inoculated on petri plates containing sterilized skim milk agar media. The plates were kept inverted in incubator at different temperatures of 5°C, 10°C,
15°C and 20°C for 72 hours of incubation period. Screening of the isolate was done by measuring average diameter of clear zone around the colonies [8].

Preparation of selected bacterial inoculums

The loop full of each pure bacterial culture preserved on nutrient agar slants were separately inoculated in 5ml of sterilized nutrient broth media supplemented with skim milk powder in screw capped culture tubes at pH of 7.0. The culture tubes were kept in shaking incubator at 20°C and 20rpm for 24 hours.

Preparation of submerged fermentation media and crude enzyme supernatants

1ml of each inoculum was inoculated in 50ml sterilized fermentation media containing nutrient broth supplemented with 1.4% skim milk powder in 100ml Erlenmeyer flasks. The flasks were kept in incubating shakers at different temperatures of 5°C, 10°C, 15°C and 20°C and 200rpm for 72 hours. On completion of fermentation process each flask was withdrawn and subjected to centrifugation at 10000 rpm at 4°C. The clear supernatants obtained acted as crude enzyme extracts and were used for other studies [9].

Assay of protease

The protease activity was determined by following modified casein-folin method [10] . The substrate for the process was prepared as 1% casein in 0.5M phosphate buffer at pH 7.0. Then 1ml of substrate was taken in screw caped test tube and to this 1ml of enzyme extract was added following incubation at 20°C for 60 minutes. After the completion of incubation period the reaction was stopped by adding 3ml of Trichloroacetic acid following centrifugation at 8000rpm for 10minutes. After that 0.5 ml of supernatant was pipetted out and to this 2.5ml of sodium carbonate (0.5M) was added, shaked well and kept in incubation for 20 minutes. Followed by the addition of folin phenol reagent a blue solution was produced and its absorbance was measured at 660nm against a reagent blank. The amount of amino acid released was estimated by using a tyrosine standard curve (10-100µg/ml) [11] . The protease activity was measured in terms of international units (U) that is defined as amount of enzyme that produces 1µmol tyrosine ml⁻¹min⁻¹. The enzyme activity was calculated by following formula:
Units/ml = \( \frac{\mu \text{ mol tyrosine equivalents released} \times \text{total volume of assay}}{\text{volume of enzyme} \times \text{time of assay} \times \text{volume used in calorimetric determination}} \)

All the assays were analyzed in triplicates.

**Identification of isolated bacteria**

**Morpho-biochemical characterization**

The selected bacteria were identified on the basis of morphological characteristics, cell features and biochemical characterization by following Bergey’s manual of systematic bacteriology [12].

**Molecular characterization**

The efficient isolate was subjected to molecular characterization and identification was based on 16SrRNA sequence analysis. The grown isolate in enrichment broth medium was centrifuged for 2 minutes at 13,000×g and the culture medium was removed. Deoxy ribose nucleic acid (DNA) was extracted by using HiPurA™ Bacterial Genomic DNA Purification Kit and the extracted DNA was preserved at -80°C for further analysis. The amplification of 16SrRNA of the genomic DNA was carried out by using bacterial universal primers 27F (5’AGAGTTTGATCCTGGCTCAG 3’) and 1492R (5’GGTTACCTTGTTACGACTT-3’). The polymerase chain reaction (PCR) of isolated DNA was carried out in a thermocycler (Bioneer, Korea). A final volume of 50µL of PCR reaction mixture was prepared containing 1µL of template (required DNA), 2µL of primers forward and reverse 20 picomole/µL, 1µL dNTP mix (10 mM), 3 µL MgCl₂ (25 mM), 5 µL tris-buffer (10X), 0.3 µL Taq DNA polymerase (5 units/µL) and 38 µL dionized sterilized water. PCR mixture was gently mixed and centrifuged for a few seconds. The PCR conditions for the reaction mixture after thermo-cycling were as follows: Denaturation (at 94°C and 5 minutes) and then followed by 30 cycles of 94°C for 1 min, annealing at 55°C for 45 seconds, extention at 72°C for 45 seconds and final extension at 72°C for 45. The electrophoresis of amplified PCR products was carried on agarose gel prepared by dissolving 1.5 g of agarose and 2-5 µL of ethidium bromide in 100ml Tris Acetate-EDTA.
buffer (TAE) heating the solution on an oven. A 1500 bp DNA ladder was used as molecular
weight size markers. The electrophoresis was performed for approximately 45 min at 80 mA and
visualized. High performance UV transilluminator (Bioneer, Korea) was used for the observation
of polymorphic bands and images of the gel were observed. For the purpose of sequencing the
amplified PCR product was sent to Sci-Genom Labs Pvt. Ltd., Cochin, Kerala, India. By using
the Basic Alignment Search Tool (BLAST) the unknown organism was identified using the
maximum aligned 16SrRNA sequences available in the GenBank of National Centre for
Biotechnology Information (NCBI).

**Statistical analysis**
The enzyme activity of each isolate was performed in triplicates and the results were reported as
mean. The complete random design soft-wear was used to calculate the standard error and
critical difference and the values were considered significantly different from each other at $P \leq 0.05$.

**Results**

**Isolation of protease producing bacteria**
A total of 8 morphologically different proteolytic bacteria appeared on skim milk agar by
forming zone of hydrolysis (Fig.2.a) and their pure cultures are shown in Fig-2b.

**Screening of isolated proteolytic bacteria**
On the basis of qualitative analysis the isolates showed a diversified protease activity and the
results are presented in Table-1 and Fig-2. The hydrolysis of the protein media by the efficient
isolate at different incubation periods is shown in Fig-3.

From Table-1 it is evident that the isolates showed increased protease activities by producing
larger hydrolysis zones (6 to 18 mm) as the temperature increased from 5 to 20°C. The isolate
PB$_8$ was most efficient with significantly highest protease activity by producing largest
hydrolysis zone of 18mm at 20°C after 72hours (Table-1 & Fig-3).
Mass bacterial cultures and crude enzyme extracts

All the bacteria were grown in nutrient broth media and the isolates showed a dense growth at different temperatures ranging from 5 to 20°C after 72 hours of incubation period as shown in Fig-4. However, the most dense growth was shown at 20°C by all the isolates. This might be due to the favourable conditions that support the maximum growth of bacterial isolates.

Quantitative estimation of protease activity

On the basis of primary (qualitative) screening, all the isolates were selected for quantitative estimation of protease activity and the results are shown in Table 2.

From the Table-2 it is evident that all isolates were significant with respect to protease activity over the control (0.0011 Uml⁻¹). All isolates showed minimum protease activity at 5°C that ranged from 0.5 Uml⁻¹ to 1.3 Uml⁻¹. The maximum activity was shown at 20°C by all isolates and the values ranged from 1.330 Uml⁻¹ to 2.22 Uml⁻¹. However, the highest activity of 2.22 Uml⁻¹ was shown by the isolate PB₂ followed by PB₈, PB₁, PB₄, PB₃, PB₆, PB₅ and PB₇ with enzyme activities of 2.05 Uml⁻¹, 1.74 Uml⁻¹, 1.66 Uml⁻¹, 1.63 Uml⁻¹, 1.59 Uml⁻¹, 1.55 Uml⁻¹ and 1.33 Uml⁻¹ respectively.

Identification of isolated bacteria

The potential isolate was sub-cultured on skim milk agar media to get the individual colonies as shown in Fig-6. The morphological, biochemical and molecular characteristics of the isolates is shown in Tables 3.

Based on morphological and biochemical tests the isolate exhibited the characteristics that were indistinguishable from those demonstrated by Bergey’s Manual of Bacteriology and was identified as Pseudomonas sp.
Discussion

Kashmir valley is a temperate climatic region and during cold climatic conditions the management of municipal solid waste becomes a challenge due to lower microbial activities. The major fraction of the waste is kitchen waste that supports the growth of psychrotrophic proteolytic bacteria that could be used for the cold composting of the waste to increase the nitrogen content of the final end product. In the present study a total of 8 different psychrotrophic bacteria were isolated at lower temperatures that was indicated by the formation of hydrolysis zones on the media. The presence of proteolytic bacteria in the waste might be due to the presence of protein rich kitchen waste [1, 2]. Further the formation of hydrolysis zone at lower temperature could be attributed to the psychrotrophic nature of isolates and the production of extracellular proteases [13, 4]. Proteolytic bacteria were also isolated by other researchers from the landfill soils and biodegradable portion of municipal solid wastes at higher temperatures [1, 2, 14]. The findings of the present study thus shows that proteolytic bacteria can grow in a wide range of temperature.

The isolates were subjected to screening for protease activities and it was found that the isolates produced varying amounts of protease on the test media. The isolate PB2 was considered most efficient with maximum hydrolysis zone diameter of 18mm at 20°C and the size of hydrolysis indicates the production of extracellular proteases. The protease activity of the isolates at lower temperatures might be due to the presence of cold shock and cold acclimatized proteins that allows the growth and functionality of the isolates under these conditions [15]. [14] reported proteolytic activity of various bacterial isolates that produced hydrolysis zones of diameters ranging between 2-35 mm at different incubation periods of 24 hours, 48 hours and 72 hours. [16] screened thermotolerant proteolytic bacteria at incubation periods of 24 hours, 48 hours and 72 hours and reported hydrolysis zone diameters ranged between 2 to 30mm. The efficient isolate of the present study was outstanding that showed remarkable protease production potential at lower temperatures that reflects their high biotechnological demand.

The isolates were grown in nutrient broth media for mass multiplication and production of proteases at different temperatures and the most dense growth was shown at 20°C by all the
isolates. This might be due to the favorable conditions that supports the maximum growth of bacterial isolates.

Further the quantitative estimation of protease activity revealed that all isolates were outstanding and the isolate PB$_2$ showed highest activity of 2.22U/ml. The quantitative protease activities of the isolates was found in accordance with hydrolysis zone diameters produced by the isolates. In previous studies [17] reported protease activity of different isolates varying from 0.01 to 0.43U/ml at 37°C after 48 hours of incubation period. Similarly in another study [18] studied the characterization of cold active proteases and reported highest protease activity at 15°C. Thus it was concluded that the isolates of the present study showed great potential for the production of proteases at lower temperatures that has biotechnological and industrial values.

Based on morphological and biochemical tests the isolate exhibited the characteristics that were indistinguishable from those demonstrated by Bergey’s Manual of Bacteriology and was identified as Pseudomonas sp. In previous studies the morphological and biochemical characterization identified the isolates as Bacillus sp [19] Pseudomonas sp [20]. However, molecular characterization of isolates is preferred over morphological and biochemical tests for the reason of being the best identification method [21,22]. On the basis of molecular characterization and 16SrRNA sequence analysis the efficient isolate (PBLF3) was 96% similar to Pseudomonas fluorescense with accession no. MH266217. In previous studies [23] reported psychrotolerant Bacillus cereus, [24] reported Bacillus subtilis and [25] reported psychrotrophic Pseudomonas fluorescense.

**Conclusion**

From this investigation it was revealed that the landfill site houses a large number of psychrotropic proteolytic bacteria. The bacteria showed a good potential for the production of proteases at lower temperature. On molecular level the efficient isolate PB$_2$ was identified as Pseudomonas fluorescense. The isolates reflected a starring potential for their applicability in cold composting of accumulating municipal solid waste in temperate regions like Kashmir valley and could have biotechnological and industrial demand.
Acknowledgement
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Conflict of interest
No conflict of interest declared by the authors

References


Table 1 (on next page)

figures and tables
Fig-1. Location of active cell (3) of landfill site at Achan in Srinagar

Fig-2: Isolation (a) and pure cultures (b) of proteolytic bacteria
Fig-3: Hydrolysis zones produced by isolates at lower temperatures

Hydrolysis zone after 24 hours

Hydrolysis zone after 48 hours
Hydrolysis zone after 72 hours

Fig-4: Efficient bacterial isolate showing varied hydrolysis zones at 20°C after different incubation periods

Fig-5: Production of mass bacterial cultures for enzyme extracts
Fig-6: Pure cultures of efficient isolated proteolytic bacteria
Table 1-Bacterial isolates with mean zone diameter (mm) of hydrolysis zones at different temperatures

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<th>Temperature</th>
<th>Isolate</th>
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<th>$PB_2$</th>
<th>$PB_3$</th>
<th>$PB_4$</th>
<th>$PB_5$</th>
<th>$PB_6$</th>
<th>$PB_7$</th>
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<td>9</td>
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<td>10</td>
<td>6</td>
<td>18</td>
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Mean B: 0 9.00 13.00 7.6 8.8 6.6 6.00 4.00 11.00

Factors: CD($\leq 0.05$)
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### Table 2: Quantitative protease activity of bacterial isolates at different temperatures

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### Table 3- Morphological, Biochemical and Molecular Characteristics of efficient isolate

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Table 3- Morphological, Biochemical and Molecular Characteristics of efficient isolate

Isolate Morphological characteristics

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<tr>
<th>Colony features</th>
<th>Cell features</th>
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<td>Grams nature</td>
</tr>
<tr>
<td>Colony shape</td>
<td>Cell shape</td>
</tr>
<tr>
<td>Margin</td>
<td>Translucent</td>
</tr>
<tr>
<td>Elevation</td>
<td>Gram - rods</td>
</tr>
<tr>
<td>Surface</td>
<td>Smooth</td>
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PB2

- Yellow
- green
- Round
- Entire
- Thick leafy
- Translucent
- Gram - rods
- Smooth
### Biochemical characteristics

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<tr>
<th>Indole</th>
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### Molecular characteristics

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