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1 **Isolation and Characterization of Psychrotrophic Proteolytic Bacteria from Landfill Site**
2 **under Temperate Climatic Conditions of Kashmir Himalaya**
3
4 Basharat Hamid[1], Arshid Jehangir[2], Zahoor Ahmad Baba[3], Muneer Ahmad Wani[4], Imran Khan[5]
5
6 [1, 2, 4, 5] Department of Environmental Science, University of Kashmir, Hazratbal Srinagar-19006,
7 India
8 [3]Biofertilizer Research Laboratory, Division of Basic Science and Humanities, Wadura, Sher-e-
9 Kashmir University of Agricultural Sciences and Technology, FOA Sopore-193201,India.
10
11 Corresponding Author:
12 Basharat Hamid
13 Shirpora Pattan, Srinagar, Jammu and Kashmir, 193121, India
14 Email address: basharathamid73@gmail.com
15
16 **Abstract**
17 The temperate climatic regions face the problem of waste accumulation due to lower
18 environmental temperatures. However, these regions harbor cold active microbes viz.
19 psychrotrophic proteolytic bacteria that play an important role in the degradation of protenaceous
20 materials of the waste stream. Hence in the present study psychrotrophic proteolytic bacteria
21 were isolated from waste samples collected from landfill site by using random sampling method
22 under environmental temperature of 10°C. By using serial dilution and spread plate technique a
23 total of 8 morphologically different psychrotrophic proteolytic bacteria were isolated on skim
24 milk agar media at pH of 7.0 and temperature of 10°C after 48hours. Under in-vitro conditions
25 all the isolates produced significant quantities of protease over the control and diameters of
26 hydrolysis zones ranged between 2 to 18 mm at temperature range of 5 to 20°C and after 72
27 hours. The corresponding quantitative protease activities of the isolates was significant that
28 ranged between 0.5 to 2.25 U/ml and the isolate PB_2 was most efficient with highest protease
29 activity of 2.25U/ml at 20°C.The best isolate also showed hydrolysis of other protenaceous
30 substrates (gelatin and elastin).The enzyme produced was purified to 18.45folds and the
31 molecular weight of purified enzyme was estimated to be 55kDa by SDS-PAGE analysis.
32 Further protease inhibition analysis revealed that extracellular proteases secreted by the isolate
33 PB_2 were mainly serine and metallo-proteases. Based on 16SrRNA analysis the isolate was
34 identified as *Pseudomonas florescence* with 96% similarity. The study represents the first
35 comprehensive analysis on isolation of cold active protein degrading bacteria from municipal

36 solid waste landfill site. The isolate *Pseudomonas florescence* could be used for enhanced
37 decomposition of a variety of protenaceous substrates at lower temperatures and could have
38 industrial application for producing many important proteases.
39
40
41 **Introduction**
42 In developing countries the growing population, rising economic conditions, rapid urbanization
43 and the rise in community living values have greatly accelerated the municipal solid waste
44 production rate [1] Of different kinds of MSW management techniques, land-filling has proved
45 to be very cost effective managing about 80% of the MSW worldwide [2]. In most developing
46 countries the highest percentage (40– 70%) of MSW consists of organic matter which is able to
47 retain a high moisture content [3, 4, 5]. The presence of organic materials in the waste stream
48 also contains protein rich kitchen waste that supports the growth of proteolytic bacteria [6, 7]
49 and thus landfill sites are regarded as store houses of effective waste degrading bacteria. The
50 bacterial cells consume the wastes of different types by producing various specific enzymes.
51 In temperate climatic regions the decomposition biodegradable waste is retarded during cold
52 temperatures due to slowing down of microbial activities. But these regions contain some
53 psychrotrophic protein degrading bacteria that could be used for decomposition of protenaceous
54 materials of the waste. Hence the interest was developed to study the nature of extracellular
55 enzymes secreted by these cold adapted bacteria.
56 Psychrotrophic bacteria are the bacteria that grow at lower temperatures (5°C) and had higher
57 optimal growth temperatures [8]. The psychrotrophic microorganisms are not present only in
58 permanently cold areas but such microorganisms are also present in seasonally cold
59 environments because of their ability to tolerate lower temperatures [9]. In cold environmental
60 conditions psychrotrophs are believed to play an important role in the biodegradation of organic
61 matter [10, 11]. The bacteria that are adapted to lower temperatures have special type of proteins
62 called cold acclimatized proteins (Caps) that are involved in important metabolic function(s) at
63 low temperature by maintaining membrane fluidity and/or by replacing cold-denaturized
64 peptides [13].
65
66 A protease is an enzyme that carries out the hydrolysis of peptide bonds between the amino acids
67 occurring in a polypeptide chain forming the protein and cause their splitting into free amino

68 acids [14] .Proteases have found applications in diverse fields such as detergent industry, leather
69 processing, silk degumming, food and dairy, baking, pharmaceutical industries, silver recovery
70 from x-ray films, waste management and others. This high demand of proteases has triggered the
71 scientific community to isolate novel strains and optimize the process parameters for the
72 production of proteases [15]. The production of bacterial proteases depends on number factors
73 like type of strain, medium composition, cultivation method, nutrient requirement, metal ions,
74 pH, and temperature, time of incubation and thermo stability [16]. Most commonly used
75 methods for the identification of potential enzyme producing isolates involves the morphological
76 and biochemical methods, however, the technique of 16SrRNA sequencing appears to have
77 potential ability to differentiate strains at the subspecies level [17].
78 Kashmir valley is a temperate climatic region and management of municipal solid waste
79 becomes a major issue during cold environmental conditions due to slowing down of microbial
80 activity. Hence the interest was developed to set the aim of isolation and characterization of
81 psychrotrophic proteolytic bacteria that could be used for the development of efficient bacterial
82 consortium for rapid composting of biodegradable solid waste. Further no such work that
83 involved the isolation of cold tolerant bacteria from landfill site has been done to the best of our
84 knowledge and characterization of the isolates would serve as a base line data for characteristics
85 of cold adapted protease producing microorganisms.
86
87 **Materials & Methods**
88
89 **Collection of samples**
90 By using random sampling method waste samples were collected during winter season from
91 Achan landfill site that lies between 34° 7' 00" North and 74° 47' 38.08" East (Fig-1). The waste
92 samples mixed with soil were collected aseptically and transported intact at ambient temperature
93 in sealed polythene bags to the laboratory for analysis.

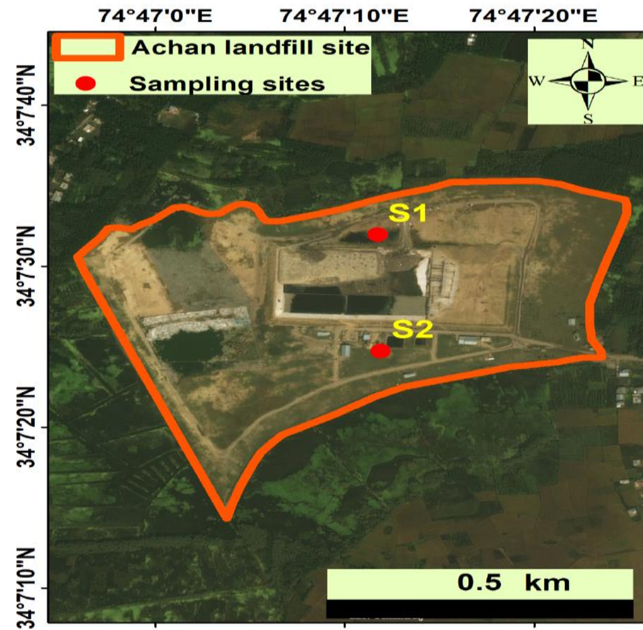


Fig-1. Location of active cell (3) of landfill site at Achan in Srinagar

Isolation of psychrotolerant protease producing bacteria

The isolation of bacteria was carried on Skim milk agar media by serial dilution and spread plate technique [18]. The samples were serially diluted up-to the dilution of 10^{-6}. 1ml solution from last three dilution tubes was transferred on solid sterilized petriplates containing Skim Milk agar media. The plates were incubated at ambient environmental temperature of 7°C for 48 hours. After the completion of incubation period the plates were observed and some of the bacterial isolates showed formation of hydrolysis zone around the respective colonies and these isolates were considered positive for proteases. The bacterial colonies were picked based on difference in morphology, size and color and sub-cultured to achieve the pure cultures and preserved in nutrient agar slants at 4°C.

Screening of isolated bacteria through qualitative enzyme activities

All the isolated proteolytic bacteria were screened for protease activity, as per modified protocol of [19]. The pure cultures of the isolate were inoculated on the skim milk agar media (Hi-Media), pH 7.0. The plates were incubated inverted at different temperatures of 5°C, 10°C, 15°C and 20^0C for 72 hours. After incubation period, the hydrolysis zones were observed around the growing bacterial colonies and the diameters of hydrolysis zones were recorded.

114

115 **Hydrolysis Ability of Proteases to other different Substrates**

116

117 Two other solid media were prepared by adding gelatin (0.5% w/w) and elastin (0.5% w/w)
118 powder into nutrient agar media. The efficient bacterial strain was inoculated on the two media
119 to check its ability to form clear hydrolysis zone on the screening medium were and incubated at
120 20◦C for 48 hours. The diameters of hydrolytic zone formed by the strain were measured
121 according to [20].

122

123 **Preparation of selected bacterial inoculums**

124 The loop full of each pure bacterial culture preserved on nutrient agar slants were separately
125 inoculated in 5ml of sterilized nutrient broth media supplemented with skim milk powder (0.5%
126 w/w) in screw capped culture tubes at pH of 7.0. The culture tubes were kept in shaking
127 incubator at 20°C and 200rpm for 24 hours.

128

129 **Preparation of submerged fermentation media and crude enzyme supernatants**

130 1ml of each inoculum was inoculated in 50ml sterilized fermentation media containing nutrient
131 broth supplemented with 1.4% skim milk powder in 100ml Erlenmeyer flasks. The flasks were
132 kept in incubating shakers at different temperatures of 5°C, 10°C, 15°C and 20°C and 200rpm for
133 72 hours. On completion of fermentation process each flask was withdrawn and subjected to
134 centrifugation at 10000 rpm at 4°C. The clear supernatants obtained acted as crude enzyme
135 extracts and were used for other studies [21].

136

137 **Assay of protease**

138 The protease activity was determined by following modified casein-folin method [22] .The
139 substrate for the process was prepared as 1% casein in 0.5M phosphate buffer at pH 7.0. Then
140 1ml of substrate was taken in screw caped test tube and to this 1ml of enzyme extract was added
141 following incubation at 20°C for 60 minutes. After the completion of incubation period the
142 reaction was stopped by adding 3ml of Trichloroacetic acid following centrifugation at 8000rpms
143 for 10 minutes. After that 0.5 ml of supernatant was pippetted out and to this 2.5ml of sodium
144 carbonate (0.5M) was added, shaked well and kept in incubation for 20 minutes. Followed by the
145 addition of folin phenol reagent a blue solution was produced and its absorbance was measured

146 at 660nm against a reagent blank. The amount of amino acid released was estimated by using a
147 tyrosine standard curve (10-100μg/ml) [23]. The protease activity was measured in terms of
148 international units (U) that is defined as amount of enzyme that produces 1μmol tyrosine ml^{-1}
149 min^{-1}.The enzyme activity was calculated by following formula:

$$\text{Units/ml} = \frac{\mu\text{ mol tyrosine equivalents released } \times \text{total volume of assay}}{\text{volume of enzyme} \times \text{time of assay} \times \text{volume used in calorimetric determination}}$$

150

151 All the assays were analyzed in triplicates.

152

153 **Diversity of extracellular Proteases produced by the efficient strain**

154

155 The efficient protease-producing strain was grown in liquid screening medium at 15◦C for 48
156 hours and centrifuged at 10000 rpm for10 minutes at 4°C. The cell free supernatant properly
157 diluted with 0.5M phosphate buffer (pH 7.0) was incubated with 1.0mM phenylmethylsulfonyl
158 fluoride (PMSF, Sigma), 1.0mM 1, 10-phenanthroline (OP, Sigma), 10mM iodoacetic acid
159 (Sigma), or 0.1mM Pepstatin A (Merk) at 20◦C for 20min. At the end of incubation, the residue
160 protease activity of every sample was measured as previously described [10]. The activity of
161 control (without inhibitor) was set as 100% and the relative activity (%) of the samples was
162 calculated. The inhibition ratio was taken as the result of the control activity minus the relative
163 activity of a sample [20].

164

165 **Partial purification of protease**

166

167 The cell free supernatant portion containing the enzyme was precipitated by ammonium sulphate
168 (up to 80% saturation) and kept overnight at 4°C. Then the mixture was centrifuged at 4°C and
169 25,000 g for 15 minutes and was the re-suspended in minimum quantity of 0.05 M phosphate
170 buffer (pH 7.0) and followed by its dialysis against the same buffer. This dialysate was tested for
171 homogeneity and used for further characterization studies.

172

173 **SDS – PAGE and Zymography**

174

175 SDS PAGE (10%) was performed according to the protocol described by [24], under reducing
176 conditions. The molecular weight of purified protease was calculated on the basis of semi

177 logarithmic plots of the mobility of the band on SDS-PAGE versus broad range standard
178 molecular weight markers (28, 39, 60, 84 120 and 215 kD) . The samples were applied in non-
179 reducing Laemmli buffer without heat denaturation and run at 90Volts. The gel was washed in
180 2.5% Triton- X- 100 (3×15 min) to remove SDS. Protein bands were located by staining the gel
181 with Coomassie Brilliant Blue R-250.

182

183 **Morpho-biochemical characterization of the bacterial strain**

184 The selected bacteria were identified on the basis of morphological characteristics, cell features
185 and biochemical characterization by following Bergey's manual of systematic bacteriology [25]**.**
186 The biochemical tests included Indole test, Methyl red test, Citrate utilization test, Oxidase test,
187 Catalase test, Casein hydrolysis test, Starch hydrolysis test, Urease test, Vogesproskuer test.

188

189

190 **Molecular characterization**

191 The isolate showing the highest protease activity was subjected to molecular characterization and
192 identification was based on 16SrRNA sequence analysis. The grown isolate in enrichment broth
193 medium (skim milk broth) was centrifuged for 2 minutes at 13,000×g and the culture medium
194 was removed. Deoxy ribose nucleic acid (DNA) was extracted by using HiPurA^{TM} Bacterial
195 Genomic DNA Purification Kit and the extracted DNA was preserved at -80°C for further
196 analysis. The amplification of 16SrRNA of the genomic DNA (450 pico moles) was carried out
197 by using bacterial universal primers 27F (5′AGAGTTTGATCCTGGCTCAG 3′) and 1492R
198 (5′GGTTACCTTGTTACGACTT-3′) [26]. The polymerase chain reaction (PCR) of isolated
199 DNA was carried out in a thermocycler (Bioneer, Korea). A final volume of 50µL of PCR
200 reaction mixture was prepared containing 1µL of template (required DNA) , 2µL of primers
201 forward and reverse 20 picomole/µL, 1µL dNTP mix (10 mM) , 3 µL $Mgcl_2$ (25 mM) , 5 µL tris-
202 buffer (10X) , 0.3 µL Taq DNA polymerase (5 units/µL) and 38 µL dionized steriled water. PCR
203 mixture was gently mixed and centrifuged for a few seconds. The PCR conditions for the
204 reaction mixture after thermo-cycling were as follows: Denaturation (at 94°C and 5 minutes) and
205 then followed by 30 cycles of 94°C for 1 min, annealing at 55°C for 45 seconds, extention at
206 72°C for 45 seconds and final extension at 72°C for 45 seconds. The electrophoresis of amplified
207 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).

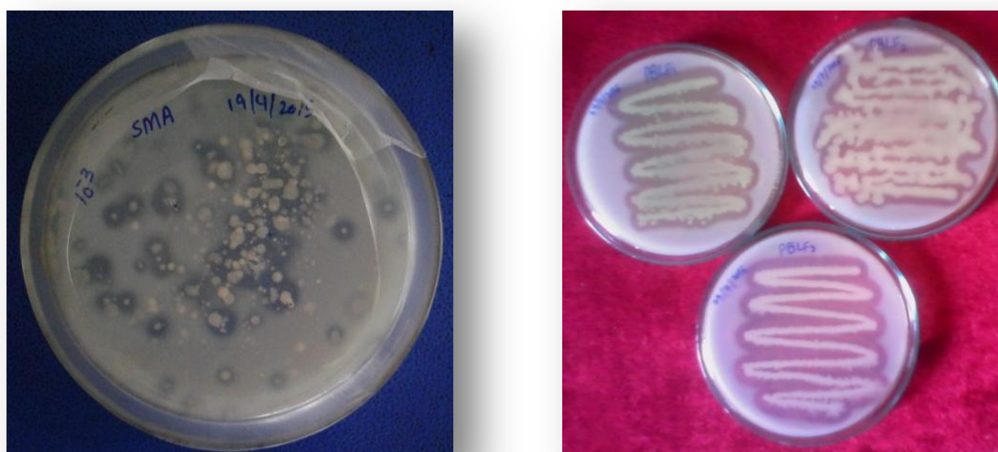
Statistical analysis

The enzyme activity of each isolate was performed in triplicates and the results were reported as mean. The complete random design 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

The results of isolation revealed that a total of 8 morphologically different proteolytic bacteria appeared on skim milk agar by forming zone of hydrolysis (Fig.2A) .Further the isolates were pure cultured as shown in Fig-2B.



a. **b.**

Fig-2: Isolation (A) and pure cultures (B) of psychrotrophic proteolytic bacteria

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. The isolates formed good hydrolysis zones on skim milk agar media after 24 hours of incubation (Fig-3), however the hydrolysis of the protein media by the efficient isolate at different incubation periods is shown in Fig-4.

From Table-1 it is evident that the isolates showed increased protease activities by producing larger hydrolysis zones (6mm to 18 mm) as the temperature increased from $5^{o}C$ to $20^{o}C$. The isolate PB_2 was most efficient with significantly highest protease activity by producing largest hydrolysis zone of 18mm at $20^{o}C$ after 72hours (Table-1 & Fig-4).

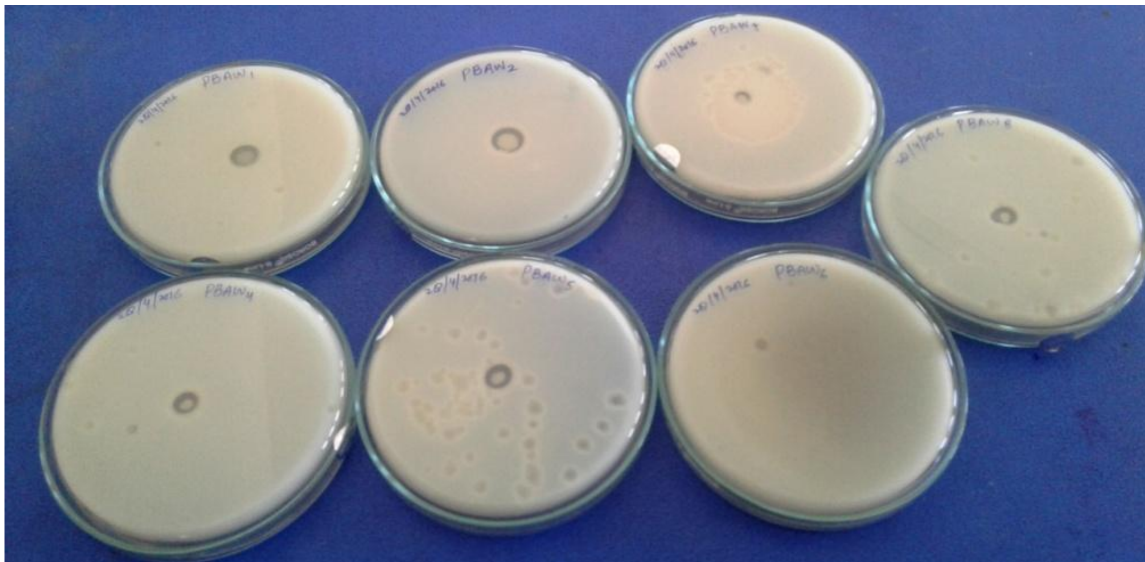


Fig-3: Hydrolysis zones produced by isolates at lower temperatures

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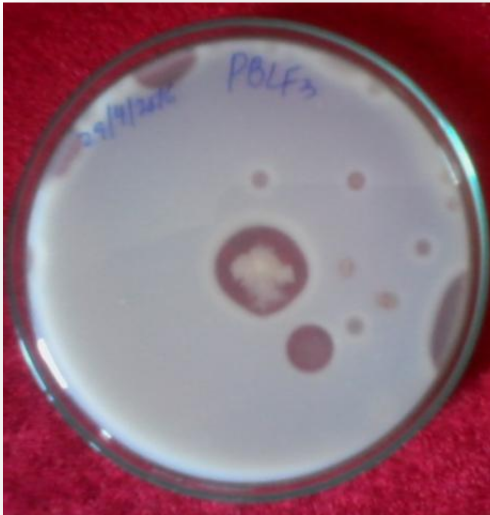

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247 **Hydrolysis zone after 24 hours** **Hydrolysis zone after 48 hours**

248

249 **Hydrolysis zone after 72 hours**

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

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260 **Table 1-Bacterial isolates with mean zone diameter (mm) of hydrolysis zones at different temperatures**

261

Temperature	Isolate								
	•P_0	•PB_1	•PB_2	•PB_3	•PB_4	•PB_5	•PB_6	•PB_7	•PB_8
5°C	0	6	9	6	7	5	7	2	8
10°C	0	10	13	7	9	6	8	4	11
15°C	0	11	15	10	11	8	10	6	13
20°C	0	13	18	11	12	9	10	6	16
SE(±)	0	0.05	0.06	0.05	0.06	0.05	0.06	0.02	0.06
CD(p≤0.05)	0	0.19	0.41	0.17	0.21	0.16	0.20	0.18	0.37

262
263 • Mean value
264 SE =standard error
265 CD= critical difference
266

267 **Hydrolysis of other different substrates**

268 The efficient bacterial isolate (PB_2) was tested for its ability of hydrolyzing different other
269 proteins by measuring the diameter of hydrolysis zones around the colonies on the plates
270 containing gelatin and elastin. From the results it was observed that the isolate could hydrolyze
271 both proteinaceous substrates, however the hydrolysis was much better seen in gelatin media as
272 shown in Table 2.
273 In all, difference in the hydrolysis ability to the three proteins (skim milk agar, gelatin and
274 ealstin) of the extracellular protease from the screened strain, inferred from the variation of
275 diameter of hydrolysis zone, reflected its difference in kind toward the three proteins.
276
277
278

Table 2: Diameters hydrolysis zone on different protein substrates

Strain	Hydrolysis zone (mm)	
	Gelatin	Elastin
PB_2	•16.1	•2.1

Where, mm represents millimeter

• represents mean value

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-5. However the most dense growth was shown at 20°C by all the isolates. This might be due to the favorable conditions that support the maximum growth of bacterial isolates.

Fig-5: Production of mass bacterial cultures for enzyme extracts

294
295
296
297 **Quantitative estimation of protease activity**

298 On the basis of primary (qualitative) screening, all the isolates were selected for quantitative
299 estimation of protease activity and the results are shown in Table 3.
300
301 From the Table-3 it is evident that all isolates were significant with respect to protease activity
302 over the assay without the enzyme (control; 0.0011 Uml^{-1}). All isolates showed minimum
303 protease activity at 5°C that ranged from 0.5 Uml^{-1} to 1.3 Uml^{-1}. The maximum activity was
304 shown at 20°C by all isolates and the values ranged from 1.330 $Uml^{-1}$to 2.22 Uml^{-1}.However, the
305 highest activity of 2.22 $Uml^{-1}$was shown by the isolate PB_2 followed by PB_8, PB1, PB4, PB3,
306 PB6,PB5 and PB7 with enzyme activities of 2.05 Uml^{-1}, 1.74 Uml^{-1},1.66 Uml^{-1}, 1.63Uml^{-1},1.59
307 Uml^{-1}, 1.55 Uml^{-1} and 1.33 Uml^{-1}respectively.

308 **Table 3: Quantitative protease activity of bacterial isolates at different temperatures**
309

Temperature	Isolate								
	*P_0	*PB_1	*PB_2	*PB_3	*PB_4	*PB_5	*PB_6	*PB_7	*PB_8
5°C	0.03	1.10	1.50	0.85	1.05	0.95	1.0	0.50	1.30
10°C	0.07	1.50	2.01	1.25	1.43	1.20	1.25	1.01	1.75
15°C	0.09	1.70	2.20	1.59	1.64	1.53	1.57	1.29	2.01
20°C	0.1	1.74	2.25	1.63	1.66	1.55	1.59	1.33	2.03
SE (±)	0.002	0.14	0.23	0.15	0.15	0.11	0.17	0.18	0.17
CD(≤0.05)	0.003	0.42	0.67	0.44	0.45	0.33	0.50	0.06	0.50

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311 • Mean value
312 SE =standard error
313 CD= critical difference
314
315
316 **Type of extracellular protease from the best strain**

317 The kind of extracellular proteases produced by the screened protease-producing bacteria was
318 investigated by analyzing the effects of different inhibitors on the protease activity (Table 4).
319 PMSF (serine protease inhibitor), OP (metalloprotease inhibitor), iodoacetic acid (cysteine
320 protease inhibitor), and Pepstatin A (aspartic protease inhibitor) were used to inhibit the activity
321 of the proteases secreted by the screened strain to identify the types of these proteases. From the
322 results it was observed that the isolate PB_2 produced enough protease for activity inhibition
323 analysis. Among all inhibitors , only PMSF and OP were able to inhibit the protease activity of
324 the isolate at high degree indicating that the isolate was able to produce simultaneously mainly
325 serine and metallo-protease.

326 **Table 4: Effects of inhibitors on the extracellular proteases secreted by strains**
327

Strain	[a] **Inhibition ratio (%)**		**Control**
	PMSF (1mM)	OP (1mM)	
PB_2	0	0.5	100%

328
329 [a] The activity of a sample without any inhibitor was taken as control (100%). The inhibition ratio (control activity
330 minus the relative activity of a sample with an inhibitor). PMSF, Phenylmethylsulfonyl fluoride; OP, 1,10-
331 Phenanthroline.
332
333
334
335
336
337
338 **Purification of protease and Polyacrylamide gel electrophoresis**
339
340 The extracellular protease were partially purified by ammonium sulphate precipitation followed
341 by a series of chromatography steps in order to give an overall purification. The molecular

342 weight of purified protease was calculated on the basis of semi logarithmic plots of the mobility
343 of the band on SDS-PAGE using standard molecular weight markers. From the analysis the
344 molecular weight of the purified protease was determined to be 55.0 kDa. After column
345 purification the zymogram analysis presented a single band revealing that the protease is active
346 as a monomer, hence the results indicated that the protease is a monomeric enzyme (Fig-6).
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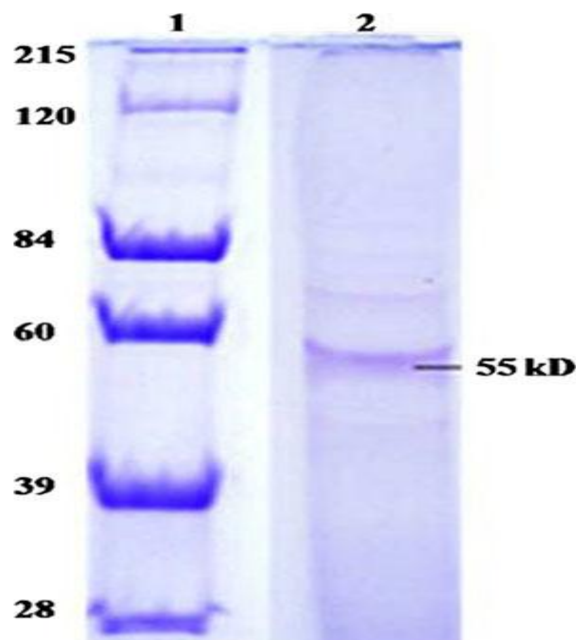

350
351 **Fig-6: SDS-PAGE of purified protease: lane 1 molecular weight**
352 **ladder; lane 2 partially purified protease**
353
354
355
356 **Identification of isolated bacteria**
357 The potential isolate was sub-cultured on skim milk agar media to get the individual colonies as
358 shown in Fig-7. The morphological, biochemical and molecular characteristics of the isolates is
359 shown in Tables 4. Based on morphological and biochemical tests the isolate exhibited the
360 characteristics that were indistinguishable from those demonstrated by Bergey's Manual of
361 Bacteriology and was identified as *Pseudomonas sp.* The results of molecular characterization
362 revealed that the isolate had 97% similarity with *Pseudomonas fluorescens*.

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364
365 **Fig-6: Pure cultures of efficient isolated proteolytic bacteria**
366
367 **Table 5- Morphological, Biochemical and Molecular Characteristics of efficient isolate**
368

Isolate	**Morphological characteristics**							
	Colony features						**Cell features**	
	Colony color	**Colony shape**	**Margin**	**Elevation**	**Surface**	**Visual characteristic**	**Grams nature**	**Cell shape**
PB2	Yellow green	Round	Entire	Thick leafy	Smooth	Translucent	Gram -	Thin rods
	Biochemical characteristics							
	Indole	**M.R**	**Citrat**	**Oxidas**	**Catalase**	**Casein**	**Starh**	**Urease**	**V.P**

		e	e		Hydrolysis	hydrolysis		
−	+	+	+	−	+	+	−	+

Molecular characteristics

Max. Score	Percent Identity	16SrRNA Sequence Length	Best match using BLASTn	Accession Number
1958	96%	834	*Pseudomonas florescence* gene for 16SrRNA	MH**266217**

369
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371
372
373 **Discussion**
374
375 Ecology of the Jammu and Kashmir is extremely fragile and the environment has taken a
376 backseat resulting in problems such as climate change, water scarcity and even solid waste
377 management. The climatic conditions of Kashmir are mostly under the influence of the cold
378 extremities for nearly two third time of the year. The colder temperature for the maximum times
379 of the year posses a greater challenge to decomposition of organic wastes as a result of reduced
380 microbial activities. Further these lower temperature conditions create technical challenges for
381 efficient composting of organic wastes. The major fraction of the waste is kitchen waste that
382 supports the growth of psychrotrophic proteolytic bacteria that could be used for the cold
383 composting of the waste to increase the nitrogen content of the final end product. In the present

384 study a total of 8 different psychrotrophic bacteria were isolated and the proteolytic activity of
385 the isolates was apparent by the formation of hydrolysis zones around the bacterial colonies. The
386 isolates were cold tolerant for the reason of growing at 5°C and showed optimum growth at 20°C
387 [8]. The presence of proteolytic bacteria in the waste might be due to the presence of protein rich
388 kitchen waste [6, 7]. Proteolytic bacteria were also isolated by other researchers from the landfill
389 soils and biodegradable portion of municipal solid wastes at higher temperatures [6, 7, 27] .The
390 findings of the present study thus shows that proteolytic bacteria can grow in a wide range of
391 temperature. The isolates were subjected to screening for protease activities and it was found that
392 the isolates produced varying amounts of protease on the test media by producing hydrolysis
393 zones. The formation of hydrolysis zone at lower temperature could be attributed to the
394 psychrotrophic nature of isolates and the production of extracellular proteases [13, 4]. The
395 qualitative protease activity (formation of hydrolysis zones) of bacterial strains was also reported
396 by other researchers [27, 28, 29]. In the present study the isolates showed remarkable protease
397 productions at lower temperatures that distinguishes them from other enzyme producing bacteria
398 that are metabolically active at higher temperatures. The isolates were grown in nutrient broth
399 media for mass multiplication and were grown in protease production media and the best results
400 were obtained at 20°C for these conditions supports the maximum growth of bacterial isolates
401 and production of proteases. The quantitative protease activities of the isolates were found in
402 accordance with hydrolysis zone diameters produced by the isolates. The protease activity of the
403 isolates at lower temperatures might be due to a number of reasons viz. the presence of cold
404 shock, cold acclimatized proteins that allows the growth and functionality of the isolates under
405 these conditions and their own adaptive capacity coping with cold stresses [30,12] Hence these
406 cold adapted strains have attracted increasingly attention with their higher catalytic enzymes at
407 cold ambient temperatures, which could provide huge biotechnological applications for the
408 practical production [31, 32].The protease activity of bacterial strains on quantitative basis were
409 also demonstrated by other researchers [33,29]. Further it was investigated that the best isolate
410 PB_2 was able to produce serine and metallo proteases as the principle proteases and this ability of
411 secreting variety of extracellular proteases can make bacteria efficiently hydrolyze diverse and
412 complex proteinaceous substances in the landfill site. As determined by SDS-PAGE analysis, the
413 partially purified protease from *Pseudomonas fluorescens* is homogenous, forming a single band
414 of about 55.0 kDa (Fig. 3). However, in previous study the molecular weight of the similar

415 bacterial species was reported as 47Kda which is close to our findings [11]. Morpho-biochemical
416 tests showed that the best isolate was identified as *Pseudomonas sp*, however 16SrRNA
417 sequence analysis declared the isolate as *Pseudomonas fluorescens*. Further molecular
418 characterization of isolates is preferred over morpho-biochemical tests for the reason of being the
419 best identification method [34, 35]. Previous investigations related to morpho-biochemical
420 characterization of the bacterial strains reported *Bacillus sp* and *Pseudomonas sp* [36, 37]. In
421 several other similar studies the psychrotolerant bacterial isolates were identified on molecular
422 basis as *Bacillus cereus*, *Bacillus subtilis* and *Pseudomonas fluorescence* [38, 39, 26].
423

424 **Conclusion**

425 From this investigation it was revealed that the landfill site houses a large number of
426 psychrotropic proteolytic bacteria. The bacteria showed a good potential for the production of
427 proteases at lower temperature. On molecular level the efficient isolate PB_2 was identified as
428 *Pseudomonas florescence* .The isolates reflected a starring potential for their applicability in cold
429 composting of accumulating municipal solid waste in temperate regions like Kashmir valley and
430 could have biotechnological and industrial demand. Our work on the bio-prospective from the
431 screened strains for novel proteases is on the way.
432

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442

443 **Conflict of interest**

444 No conflict of interest declared by the authors
445

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