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研究报告

# Purification and Properties of Cold-active Metalloprotease from *Curtobacterium luteum* and Effect of Culture Conditions on Production

Mohammed Kuddus[1], and Pramod W. Ramteke[2]

1 *Protein Research Laboratory*, *Department of Biotechnology*, *Integral University*, *Kursi Road Lucknow* 226026, *India*
2 *College of Biotechnology and Allied Sciences*, *Allahabad Agricultural Institute, Deemed University*, *Allahabad* 211007, *India*

**Abstract:** *Curtobacterium luteum*, a gram-positive psychrotrophic bacterium, secreting an extracellular protease was isolated from the soil of Gangotri glacier, Western Himalaya. The maximum enzyme production was achieved when isolate was grown in a pH-neutral medium containing skim milk at 15°C over 120 hour. The metal ions such as $Zn^{2+}$ and $Cr^{2+}$ enhanced enzyme production. The specific activity of purified enzyme was 8090 u/mg after 34.1 fold purification. The 115 kD enzyme was a metalloprotease (activity inhibited by EDTA and EGTA) and showed maximum activity at 20°C and pH 7. The enzyme was active over a broad pH range and retained 84% of its original activity between pH 6–8. There was no loss in enzyme activity when exposed for 3 hours at 4°C–20°C. However, lost 65% of activity at 30°C, and was almost inactivated at 50°C, but was resistant to repeated freezing and thawing. The enzyme activity was stimulated by manganese ions; however, it was inactivated by copper ions.

**Keywords**: enzymes, cold-active metalloprotease, *Curtobacterium luteum*, Gangotri glacier

## Introduction

During the past decade it has been recognized that Microorganisms growing at low temperature regions are important for their metabolic contribution in the ecosphere as well as for their enzymes that provide a wide biotechnological potential, offering numerous economic and ecological advantages over the use of organisms and their enzymes which operate at higher temperatures[1−6]. Cold-active enzymes are characterized by high catalytic efficiency at low and moderate temperatures at which homologous mesophilic enzymes are not active, and are thermolabile[7]. These properties of cold-active enzyme are concern for both basic research and industrial application. The application of such enzymes enables lowering of the temperature and shortening of processing times without a loss of efficiency, which leads to save energy consumption. Proteases are one of the most important industrial enzymes, and they are a convenient tool whenever protein removal is needed[8]. Cold-active enzymes have relatively low optimal temperatures hence they are potentially useful in some industrial applications such as food processing, detergent additives and biotransformation of chemicals [4]. Thus, it is desirable to search for new source of cold-active proteases with novel properties from as many different sources as possible. There are several reports on cold-active proteases produced by microorganisms from different habitat[9−15]. Comparatively little information is available on enzyme from microorganisms isolated from glacier regions that may be ideal habitat for cold adapted organisms because it consisting predominantly of organic debris such as microorganisms, pollen, plant, animal litter and waste materials from expeditions. The present study highlighted production optimization and properties of the cold-active metalloprotease produced by *Curtobacterium luteum*, isolated from soil of Gangotri glacier, India.

## 1 Methods

### 1.1 Isolation of extracellular protease producing bacterium

In order to isolate cold-tolerant bacteria, the soil

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**Corresponding author:** Mohammad Kuddus. Tel: +91-9415392163; E-mail: kuddus_biotech@yahoo.com

samples were collected from vicinity of Gangotri glacier, Western Himalaya. The glacier is situated in between 30°44′–30°56′ N and 79°04′–79°15′ E, covers an area of about 75 $km^2$ and having temperature 5°C–2°C in summer and subzero in winter. The samples were serially diluted in sterile cold saline solution and plated on skim milk agar media, and incubated at (15±1)°C. A clear zone of skim milk hydrolysis gave an indication of protease producing organisms. Depending upon clear zone diameter, one isolate was selected after repeated streaking for identification and enzyme production. The isolate was fully identified and characterized by Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India.

### 1.2 Protease production

Protease production was carried out in a medium contained (g/L): glucose, 10; peptone, 5; yeast extract, 5; $KH_2PO_4$, 1 and $MgSO_4·7H_2O$, 0.2. The pH of the autoclaved broth was adjusted by adding sterilized $Na_2CO_3$ solution (20% *W*/*V*). The above medium was inoculated at 1.0% (*V*/*V*) with 24 hour old (*OD*=0.6) culture and incubated at (15±1)°C in a refrigerated incubator shaker (Scigenics Biotech. Ltd., India) at 80 r/min for 48 hours. The growth cultures were then centrifuged at 4°C (10 000×g, 15 min) and the supernatant was used for protease assay.

### 1.3 Assay to determine proteolytic activity and protein content

Protein concentration was determined by the method of Lowry *et al*[16] using bovine serum albumin (BSA) as standard. The protease activity with azocasein (Sigma) as a substrate was assayed by the modified method of Secades and Guijarro[17]. The activity is expressed in units. One unit of enzyme activity was defined as the amount which yielded an increase in $A_{420}$ of 0.01 in 30 min at 30°C.

### 1.4 Optimization of protease production

The methods adopted to study the optimization of growth parameter for protease production aimed to evaluate the effect of a single parameter at a time and later manifesting it as standardized condition before optimizing the next parameter. The experiments were conducted in triplicate and the results are average of three independent experiments. To determine optimum time duration for cold-active protease production, the respective broth media was inoculated with the organism and incubated at (15±1)°C. The samples were withdrawn at different time intervals and the enzyme activity was assayed. To find out optimum temperature, the broth media was inoculated with the organism and incubated at different temperatures. Optimum pH condition for maximum production of protease was ascertained by inoculating the broth media having different pH. To optimize the best condition for fermentation, the broth inoculated with the organism was incubated in static condition and in a rotary shaker (120 r/min). The effect of inducers was studied by addition of different inducers (BSA, casein, skim milk, egg albumin) at the rate of 0.5%–1% to the media inoculated with the organism. To evaluate the impact of heavy metals on enzyme production, broth media was supplemented with maximal tolerance level of different metals at (15±1)°C and incubated under optimal condition for 48 hours. The heavy metals used were $Co^{2+}$, $Hg^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Mn^{+2}$ and $Cr^{2+}$ at the final concentration of 100, 10, 25, 100, 100, 50 and 150 μg/mL, respectively. The enzyme production was measured as per standard enzyme assay technique [17] as has been measured by other workers [18, 19]. All the experiments were carried out in triplicate and the results are the average of these three independent findings.

### 1.5 Enzyme purification

The fermented broth media incubated at optimized conditions was centrifuged at 4°C (10 000×g, 15 min) and the supernatant was used for further purification steps. The extracellular protease in the cell free culture supernatant was obtained by ammonium sulfate precipitation to a final concentration of 80%. The amount required for percentage saturation was calculated by the method given by Deutscher[20]. All subsequent steps were carried out at 4°C. The protein precipitate was dissolve in a minimal volume of 0.05 mol/L sodium acetate buffer and dialyzed against the same buffer for 24 hours with 6–8 changes. The fraction with maximum enzyme activity obtained from ammonium sulfate precipitation was applied to DEAE-Cellulose column (Bangalore Genei, India), which was pre-equilibrated with 0.02M sodium acetate buffer, pH 5.0. The column was eluted with a linear gradient of NaCl (0.1 to 1 mol/L) in the same buffer. A total of 40 fractions were collected and assayed for protein and enzyme activity. The fractions with high protease activities were pooled and concentrated by lyophilization (Lyophilizer MSW137, Macro Scientific Works, India) and used for characterization.

### 1.6 Characterization of purified protease

#### 1.6.1 Optimum pH and pH stability

To determine the optimum pH of purified enzyme, the activity was measured at different pH by using following buffers; citrate phosphate (pH 5–6) sodium phosphate (pH 7), Tris·HCl (pH 8) glycine-NaOH (pH 9–11). Reaction mixtures were incubated at

(20±2)°C for 30 min and the residual enzyme activity was measured. The effect of pH on stability of protease was determined by pre-incubating the enzyme without substrate at different pH values (5.0−11.0) for 1 hour at (20±2)°C. The relative activity at each exposure was measured as per standard assay procedure.

1.6.2 Optimum temperature and thermal stability

The effect of temperature on protease activity was determined by performing the standard assay procedure within a temperature range from 4 °C −50°C. To determine the protease stability with changes in temperature, the enzyme was pre-incubated at different temperatures for 3 hour and relative protease activity was assayed as per standard assay procedure.

1.6.3 Effect of metal ions and inhibitors

Purified protease was pre-incubated with different metal ions (5 mmol/L) such as $Ca^{2+}$, $K^{+}$, $Ba^{2+}$, $Zn^{2+}$, $Co^{2+}$, $Cd^{2+}$, $Hg^{2+}$, $Mg^{2+}$, $Cu^{2+}$ and $Mn^{2+}$ for 30 min at 20°C and the residual activity was measured. Separate blanks with individual metal ions were prepared. The effect of protease inhibitors such as serine protease inhibitor (phenyl methyl sulphonyl fluoride, PMSF), cysteine protease inhibitors (iodoacetate, ρ-chloromercuric benzoate) metalloprotease inhibitor (ethylene diamine tetra acetic acid, EDTA; ethylene glycol-bis (2-aminoethylether)-N, N, N′, N′-tetra-acetic acid, EGTA) and aspartic protease inhibitor (pepstatin) were investigated. After 30 min pre-incubation at (20±2)°C, residual protease activity was measured by standard assay method.

1.6.4 Electrophoresis and zymogram

The SDS-PAGE of lyophilized fraction was performed, using a mini slab gel apparatus (Bangalore Genei, India), by the method of Laemmli[21]. The protein bands were visualized by staining the gel with coomassie blue and the relative molecular mass of the protein was calculated using standard protein markers (Bangalore Genei, India) run simultaneously. The gel was analyzed with a Gel Documentation system (GeneLine, Spectronics Corporation, New York).

## 2 Results and discussion

### 2.1 Isolation and characterization of psychrotrophic bacteria

Hundred bacterial isolates obtained from twenty five soil samples of Gangotri glacier were screened for secretion of extracellular protease on milk agar media at 15°C (Fig. 1). On the basis of largest clear zone of hydrolysis at low temperature one isolate was selected as potent protease producing strain, designated as GP-1, and taken for further study. The organism showed growth at 5°C–42°C and pH range of 5–12, and was a non spore-forming gram-positive irregular rod. The organism was fully characterized and identified by Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India as a *Curtobacterium luteum* and given an accession number MTCC 7529. To the best of our knowledge this is first report on *Curtobacterium luteum* producing cold-active metalloprotease.

### 2.2 Growth conditions for protease production

The protease production was gradually increases among incubation period with highest yield over 144 hour of cultivation at (15±1)°C. However, beyond 144 hour it showed rapid decline in the enzyme production that was growth independent as shown in Fig. 2. Temperature has profound influence on protease production and growth of microorganisms. In order to determine the growth and enzyme production, the cells were incubated at 4 to 45°C in production media. The cell growth and enzyme production was found to be maximal (46.4 u/mL) at 15°C over 120 hour incubation at pH 7.0 (Fig. 3).

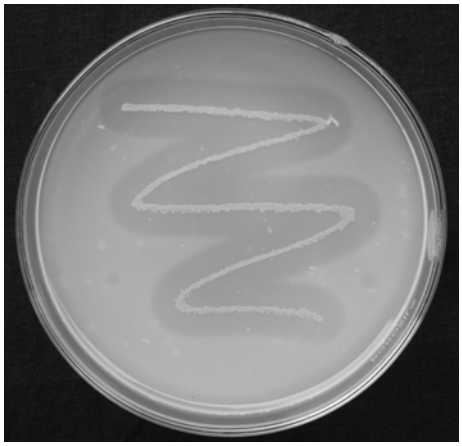

**Fig. 1 Screening of microorganisms for proteolytic activity at 15°C**

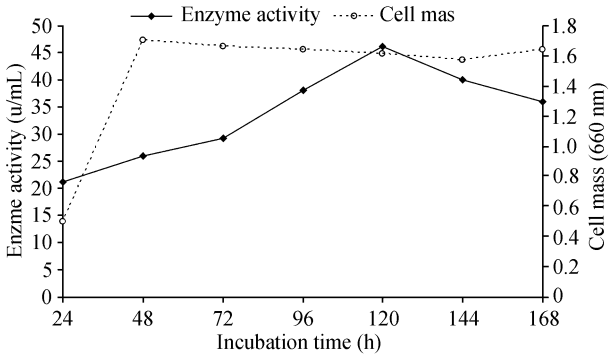


**Fig. 2 Effect of incubation time on growth and protease production at (15±1)°C**

All the other optimization experiments were performed at 15°C. According to the most widely accepted definition[22] the strain was a psychro-tolerant[22]. There was continuous decline in enzyme production with increase in incubation temperature and it was totally inhibited at 45°C. However, Dube and coworkers[23] reported maximal protease production at 30°C from five psychrotrophic bacteria isolated from lake sediments of Antarctica. The pH of culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. The influence of pH on the production of protease was studied at different pH and it was found that the isolate produced maximum protease at pH 7 and (15±1)°C (Fig. 4). Kumar and coworkers[24] reported production of neutral protease from *Bacillus* sp. S4. Therefore, it may be concluded that *C. luteum* produced neutral protease. Neutral proteases are metalloprotease, with pH optima around 7.0, which may be used in milk protein modification, nitrogen control, mash extraction and chill-haze removal in brewing, soy modification for use as flavors and in animal feeds as suggested by Schallmey *et al*.

To find out the effect of agitation on protease production, the cells were subjected to agitation at 120 r/min for 48 hour incubation at (15±1)°C. More than two fold increase in protease production was noticed in shaking condition as compared to static condition. The use of cheap sources of C and N are important as these can significantly reduce the cost of production of protease. Therefore, the utilization of various sources of carbohydrates and organic nitrogen such as BSA, casein, skim milk and egg albumin were evaluated at 15°C after 48 h incubation and agitation with respect to enzyme yield. The finding suggested that the skim milk and casein were found to be best substrates for protease production (Fig. 5). Heavy metals present in surroundings play an important role in the growth of bacteria. Impact of heavy metals on production of cold-active protease was evaluated with maximum tolerance level of metals to the organism at (15±1)°C. The enzyme production was enhanced by $Zn^{2+}$ and $Cr^{2+}$ while $Co^{2+}$ worse production. The other heavy metals such as $Hg^{2+}$, $Cd^{2+}$, $Cu^{2+}$ and $Mn^{+2}$ have no significant effect. The fluctuation in enzyme production may be due to either utilization of metals by organism or due to binding of metal ions to the enzyme that may increase or decrease enzyme activity.

### 2.3 Purification and characterization of protease

Protease was partially purified to homogeneity by precipitating with ammonium sulphate (60%) and using a single step ion-exchange chromatography on a DEAE-cellulose (Fig. 6). The enzyme was eluted out as unbound fractions (with 0.4 mol/L NaCl gradient) from the DEAE-cellulose column with 34.1 fold purification and specific activity of 8090 u/mg (Table 1).

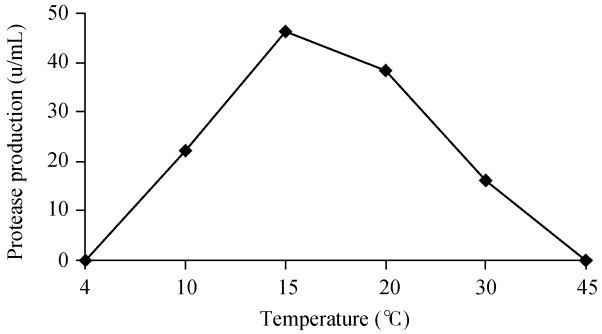


**Fig. 3 Effect of temperature on production of protease (120 h, pH 7.0)**

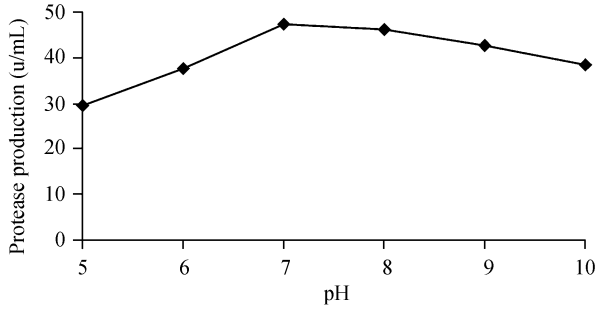


**Fig. 4 Production of extracellular protease at different pH [(15±2)°C, 120 h incubation]**

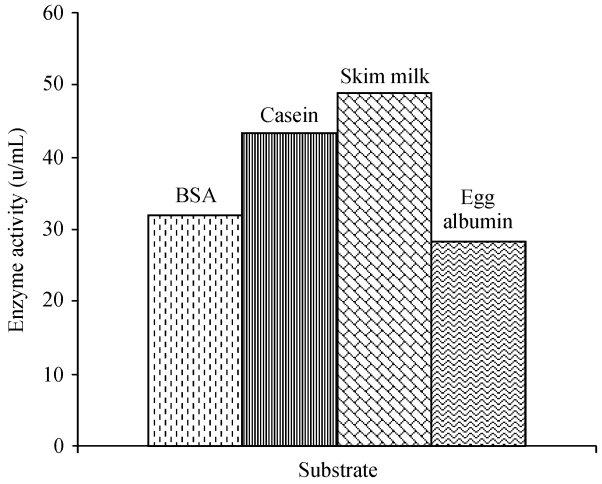


**Fig. 5 Protease production on different substrates [(15±2)°C, 48 h incubation]**

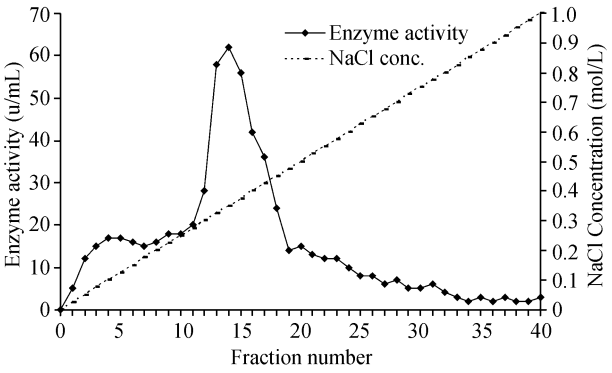


**Fig. 6 Elution profile of protease from *C. luteum* by using DEAE cellulose column**

**Table 1 Purification steps of cold active protease from *C. luteum***

| Purification step | Total activity (u) | Total protein (mg) | Sp. activity (umg) | Purified (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude enzyme | 63080.0 | 266.0 | 237.14 | – | 100.00 |
| $(NH_4)_2SO_4$ precipitation (60%) (dialyzed) | 34063.2 | 89.6 | 380.16 | 1.6 | 54.00 |
| DEAE-cellulose pool (lyophilized) | 3236.0 | 0.4 | 8090.00 | 34.1 | 5.13 |

2.3.1 pH-optima and pH-stability profile

The enzyme was active over a broad pH range (pH 5.0–11.0) and showed optimum activity at pH 7 towards casein as a substrate (Fig. 7). About 84% of the maximum activity was manifested between pH 6–8. Interestingly, 52% of the maximum activity was found at pH 5. The stability of the purified protease was determined by the pre-incubation of the enzyme in various buffers with different pH for 1 hour. The enzyme was relatively stable over a pH range of 6–8 retained 68% of the original activity at 20°C, while 95% of activity was lost after 1 hour at pH 11 (Fig. 7).

2.3.2 Temperature optima and thermal stability

The activity of purified protease was determined at different temperatures ranging from 4°C–50°C. The optimum temperature for activity was 20°C. Activity was significantly increased with temperature range of ~20°C and declined beyond 20°C with total inactivation at 50°C. High activity (33%~55% of the maximum activity) was detected at 4°C, 10°C and 30°C (Fig. 8). Most cold-active proteases display optimum activity at 30°C–45°C [15, 26]. Other investigators also reported temperature optima of 20°C for proteolytic activity[12, 27]. However, some psychrophilic bacteria produce proteases with optimum activity at 60°C[13].

The studies on enzyme stability showed that the cold-active protease was stable (retained 100% of its activity) for 3 hour at 4 °C –20°C. A similar type of result was also observed by other investigators where protease retained 100% of its maximum activity at 4 °C –20°C[14]. 65% of activity was lost at 30°C, and almost complete inactivation occurred at 50°C (Fig. 8). Thus, the enzyme was relatively stable at temperatures below 30°C. Activity of the cold-active protease was not affected after repeated freezing and thawing that may be due to adaptation of the strain to the natural habitat of Gangotri glacier where repeated freeze-thaw events are common.

2.3.3 Effect of metal ions and inhibitors on enzyme activity

The effect of various metal ions on the activity of purified enzyme at 15°C is shown in Table 2. A stronger inhibitory effect was observed in the presence of $Ca^{2+}$, $K^+$, $Ba^{2+}$, $Zn^{2+}$, $Co^{2+}$, $Cd^{2+}$, $Hg^{2+}$ and $Mg^{2+}$. Protease activity was stimulated by $Mn^{2+}$. However, $Cu^{2+}$ completely inactivated the activity at 5 mmol/L concentration. The result suggests that manganese ion apparently protected the enzyme and played a vital role in maintaining the active conformation of the enzyme[28]. A similar effect of the ion was also observed by Rahman et al. [29]. The enzyme activity was also repressed by univalent cation ($K^+$) that may be due to lose of active site conformation of the enzyme. The inhibitory effect of metal ions is well documented in the literature. Similar effects of $K^+$ on the activity of proteases were also observed by Nascimento and Martins[18].

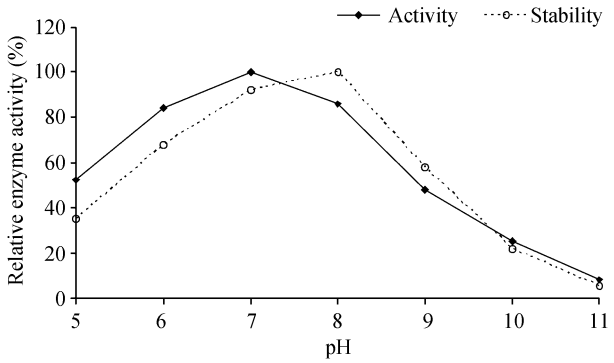


**Fig. 7 Influence of pH on the purified protease activity (Δ) and stability (■)**

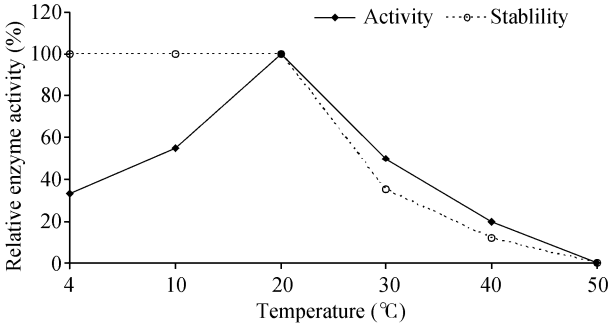


**Fig. 8 Effect of temperature on the purified protease activity (Δ) and stability (■)**

Inhibition studies primarily give an insight into the nature of active center of an enzyme. The effect of different inhibitors on the enzyme activity of the purified protease was determined (Table 2). The protease was incubated in Tris·HCl buffer (pH 8.0) with various inhibitors at 4°C for 30 min and the residual activity was measured with casein as the substrate. The enzyme

activity was almost not altered at all by 1 mmol/L of serine, cysteine and aspartic protease inhibitor. The metalloprotease specific inhibitor such as EDTA was inhibited 85%–93% of the protease activity, while EGTA exhibited 77%–88% inhibition, indicated that the enzyme belongs to the group of metalloproteases. Our findings were similar to those of Marianna *et al*[30], where the protease was inhibited (92%) by EDTA.

2.3.4 Molecular weight of the protease

The purified enzyme migrated as a single band on 10% SDS-PAGE. The molecular mass of protease obtained from *C. luteum* was determined from its mobility relative to the protein standards on SDS-PAGE by using gel documentation system. By interpolation, the molecular mass of the protease was estimated to be 115 kD, assuming that the enzyme is composed of a single polypeptide chain (Fig. 9). The proteolytic activity of this SDS-PAGE protein band was confirmed by zymogram analysis.

The exploitation of biodiversity may result in the discovery of microorganisms that produce enzymes with novel properties. Although a number of protease has been reported from microbial origins, to our best knowledge this is the first report on characterization of protease from a psychro-tolerant *C. luteum*, which showed typical feature of cold-active enzyme.

**Table 2 Effect of various metal ions and protease inhibitors on purified protease activity**

| Metal ion/ Inhibitor | Specific action | Concentration (mmol/L) | Residual enzyme activity (%) |
|---|---|---|---|
| $Ca^{2+}$ | – | 5 | 24 |
| $K^{+}$ | – | 5 | 23 |
| $Ba^{2+}$ | – | 5 | 26 |
| $Zn^{2+}$ | – | 5 | 24 |
| $Co^{2+}$ | – | 5 | 23 |
| $Cd^{2+}$ | – | 5 | 12 |
| $Hg^{2+}$ | – | 5 | 13 |
| $Mg^{2+}$ | – | 5 | 19 |
| $Cu^{2+}$ | – | 5 | 0 |
| $Mn^{2+}$ | – | 5 | 135 |
| PMSF | Serine protease inhibitor | 1 | 97 |
| | | 10 | 95 |
| EDTA | Metallo protease inhibitor | 1 | 15 |
| | | 10 | 07 |
| EGTA | Metallo protease inhibitor | 1 | 23 |
| | | 10 | 12 |
| Iodoacetate | Cysteine protease inhibitor | 1 | 92 |
| | | 10 | 87 |
| *p*CMB | Cysteine protease inhibitor | 1 | 88 |
| | | 10 | 81 |
| Pepstatin | Aspartic protease inhibitor | 1 | 94 |
| | | 10 | 86 |

PMSF: phenyl methyl sulphonyl fluoride; EDTA: ethylene diamine tetra acetic acid; EGTA: ethylene glycol-bis(2- aminoethylether)-N,N,N′,N′-tetra-acetic acid

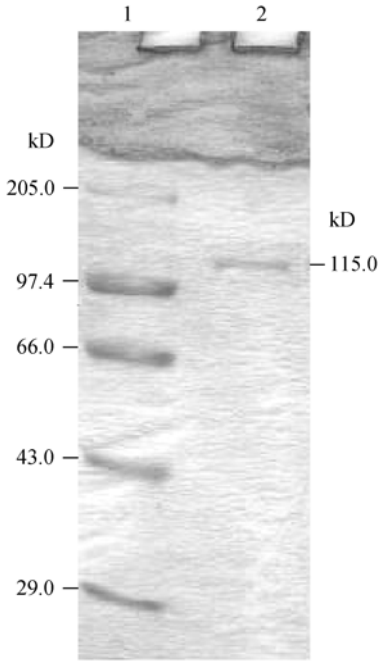


**Fig. 9 SDS-PAGE of extracellular protease from *C. luteum***
1: Standard molecular mass marker; 2: Eluted active protease

The optimum temperature for protease production from *C. luteum* was 15°C. The enzyme displayed optimum activity at 20°C and high activity at 10°C–30°C and activity was not affected by repeated freezing and thawing.Other attractive characteristics of the cold- tolerant metalloprotease are its high activity and stability at low temperatures over a broad pH range, its broad substrate specificity and its resistance to various metal ions, which could be useful properties for the possible applications in different types of industries. The *C. luteum* and its enzyme could be useful for biotransformation/biodegradation at low temperature and as new tools in molecular biology. Other potential fields of application of cold-active proteases are food processing and bioremediation in cold climates[5]. The potential of *C. luteum* to utilize a wide range of organic compounds and metal ions indicates the usefulness of such strain as an inoculums for the energy-saving bioremediation of domestic and industrial waste at ambient temperatures in cold climates, and demonstrates the biotechnological prospective of the microbial community living in extreme environments.

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