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Phylogenetic analysis of newly isolated protease producing salt tolerant psychrophilic bacteria from Tirich Mir glacier, Pakistan

Wasim Sajjad, Muhammad Rafiq, Sahib Zada, Neelam Zeb, Imran Khan, Aamer Ali Shah, Fariha Hasan*

Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan

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Abstract

Potent protease producing cold adapted bacteria were isolated from Tirich Mir glacier, Chitral, the highest mountain of the Hindu Kush range, Pakistan. Sediment, surface ice and melt water samples were collected and number of cells (CFU/ml) in samples were calculated. Casein medium was used to screen the protease producers.Three protease producing isolates; TG1-MRL, TG3-MRL and TG4-MRL were identified as *Bacillus* sp., *Serratia* sp. and *Exiguobacterium* sp. through 16S rRNA gene sequencing and their sequences were submitted to NCBI Gen Bank (Accession numbers; KF471118, KF550058 and KF550059, respectively). Optimum growth temperature and pH of all isolates were 15-25°C and 7-9, respectively. *Serratia* sp. TG3-MRL and *Exiguobacterium* sp. TG4-MRL showed growth in presence of high salt concentration, 5% and 9%, respectively. Maximum specific activity of protease was reported from *Serratia* sp. TG3-MRL (4.40 U/mg), followed by*Bacillus* sp. TG1-MRL (3.69 U/mg) and *Exiguobacterium* sp. TG4-MRL (2.42 U/mg), after 96-120 h of incubation at 15°C. Effect of pH, temperature, metal ions, inhibitors and modulators was studied on the activity of crude enzyme. All the enzymes were stable at pH 7-9. Activity of protease from *Serratia* sp. TG3-MRLwas greatly affected by Zn^{+2}. Most of the enzymes were stable in presence of EDTA, mercaptoethanol, tri-sodium citrate and PMSF. Protease by *Exiguobacterium* sp. TG4-MRL was sensitive to EDTA and PMSF. Activity of protease produced by *Bacillus* sp.TG1-MRL reduced to 20% by 1% phenyl-acetaldehyde. Stability results for protease signify their immense potential for various industrial applications such as in laundry detergent and food industries.

* **Corresponding Author:** Fariha Hasan ✉ farihahasan@yahoo.com

Introduction

Extremophiles, a dominant group of microorganisms lodging in a wide strange of natural habitats, can potentially serve in a verity of industrial application. Extremophiles contain alkaliphiles, acidophiles, psychrophiles, halophiles and thermopiles (Margesinand Schinner, 2001). During the earlier decades, the different studies and research on physiology, taxonomy and ecology of extremophiles have discovered an impressive diversity in highly stress environments. The adaptation to extreme environments, extremophiles, especially psychrophilic microbes have evolved distinctive properties, which can commercially and biotechnologically significant (Pikuta*et al.,* 2003). The Psychrophilic group of extremophiles grows well at temperatures close to the freezing point of water, but have fastest growth rates also observe up to 20°C (Madigan *et al.,* 2003). The adaptations to the low temperature depend on the ability of microbes to sense the changes in temperature. Cell membrane is one of the primary sensors to cold that act as an interface between internal and external environment (Shivaji and Prakash, 2010). Most of the studies related to psychrophiles are based on Phylogenetic analysis and only narrow attempts have been made to investigate their enzymatic potential (Dansonand Hough, 1998). The cold-adapted enzymes from cold-adapted organisms or other microbial life in extreme environments have been widely studied in recent years. The production of proteases from psychrophiles on a commercial scale is more valuable than any other enzymes use for industrial and biotechnological relevance (Niehaus*et al.,* 1999). The extracellular proteases are of commercial value and find multiple applications in various industrial sectors. Due to high catalytic activity at low temperature cold-adapted enzymes have the potency to offer novel applications for biotechnological uses(Russell, 2000). Some salt tolerant psychrotrophs have been explored that can yield extracellular proteases (Feng *et al.,* 2001). Several psychrophilic halophiles have been reported that can stand with high concentration of salt (24% NaCl), these bacteria may include *Halobacillus locisalis* (Yoon *et al.,* 2004) and *Salinivbrio costicola*(Hoover and Pikuta, 2010). In the study, one of the natural psychrophilic habitats named Tirich Mir glacier from Chitral, Pakistan was selected for the isolation and characterization of protease producing psychrophilic bacteria under different condition.

Materials and methods

Sampling

Three different samples (glacier sediments, surface ice and glacier melt water) were collected aseptically from Tirich Mir glacier, the highest peak of Hindu Kush range (Chitral). Using GPS, geographic coordinates, elevation and atmospheric pressure were measured. Temperature and pH of the sampling site was also recorded. These samples were carefully transported to Microbiology Research Laboratory, Quaid-I-Azam University, Islamabad, and stored at -20°C till further analysis.

Total viable cell count (CFU/ml)

For calculation of viable cells count and isolation of bacteria through conventional plate method, the samples were serially diluted and 100 µL of each dilutions was used to spread on the LB agar plates aseptically and incubated at 15°C for one week and CFU/mL was determined.

Screening and isolation of protease producing bacteria

Toscreen and isolate protease producing bacterial strains, all the isolates were transferred to nutrient agar medium supplemented with 1% casein soluble.

Quantitative test for protease

For the preparation of inoculum, 100 mL of the nutrient broth was prepared and inoculated a single colony from the protease positive casein agar platesin it and incubated at 15°C for 5 days. The inoculum (5%) was then transferred to the production medium (casein 1%, gelatin 2.5 g, yeast extract 1 g, peptone 2 g, NaCl 0.5 g, distilled water 500 mL, pH 7.5) and the flasks were incubated at 15°C and 120 rpm. Samples were collected after at 0 time and after every 24 hours up to 196 hours and enzyme assay was performed

routinely for quantitative analysis.

Protease assay

The proteolytic activity was determined by following a method of Kunitz (1974), using casein as substrate, which is based on the determination of split product of soluble casein known as tyrosine using tyrosine as a standard.

About 2 mL of the culture broth was taken in Eppendorf tubes and centrifuged at 10,000 rpm for 15 minutes at 4°C. The cell free supernatant (crude enzyme extract) of 1 mLwas added to 1 mL of casein substrate buffer and mixed thoroughly. The mixture was incubated for 30 minutes at 40°C. After incubation, 2 mL of 10% TCA solution was added to remove the unbound or unreacted enzymes in the form of precipitates. The tubes were then incubated at 4°C for 15 minutes. The mixture was centrifuged at 10,000 rpm for 15 minutes at 4°C. Optical density of the supernatant was measured spectrophotometrically at 540 nm (Agilent 8453 UV-visible spectrophotometer) using distilled water as a blank. Control was prepared in the same way except TCA was added before incubation.For determination of protease activity, standard curve of tyrosine was plotted. One unit of enzyme activity is defined as the amount of enzyme that liberates 1.0 µg of tyrosine per minute.

Protein Estimation

Total protein concentration of unknown sample was estimated by Lowry *et al.* (1951) method using BSA as a standard.

Production and Characterization of protease

Effect of temperature, pH, metal ions and inhibitory substances on the activity of proteases was studied.

Effect of temperature

Effect of temperature on the activity of protease was studied by incubating the crude extract at different temperatures (5, 15, 25, 35, 45, 65°C) for one hour and determined the residual activity using the method of Vazquez *et al.* (2004) with some modifications.

Effect of pH

An equal volume of the buffer solution having pH ranging from 4-11 was added to the crude enzyme extract after centrifugation of the biomass. The mixture was incubated for 3 hours at 15°C. The enzyme assay for protease was performed and residual activity was measured.

Effect of divalent metal ions, modulators, inhibitors and organic acids on protease

The cell free supernatant was incubated with an equal volumes of 10 mM solution of different metal ions, The modulators and inhibitors (1%) used were EDTA (ethylenediamine tetra-aceticacid), PMSF (Phenylmethyl sulphonyl fluoride) trisodium citrate, phenyl acetaldehyde and mercapto-ethanol for 3 hours at 15°C and residual activity was measured considering the residual activity of control as 100%.

Identification of protease producing strains

Protease producing strains selected for this study were characterized morphologically, biochemically and on molecular basis through 16S rRNA sequencing.

Molecular characterization

DNA was extracted by phenol chloroform method as described by Ausubel *et al.*(1995).

Sequencing of the isolated DNA was done using the universal 16S rRNA primers 27F(5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CTACGGCTACCTTGTTACGA-3'). For phylogenetic analysis,the sequences were compared with the 16S rRNA genes sequences in gene bank (NCBI) by using the BLAST program. By using MEGA 6 software all the obtained sequences were aligned and the phylogenetic tree were constructed by Maximum Likelihood Method with 1000 bootstrapping value in MEGA 6.0 (Tamura and Nei, 1993; Tamura *et al.*, 2013).The achieved sequences were submitted to GenBank (NCBI) and the accession numbers have been assigned.

Results

Different samples were collected from Tirich Mir glacier, Chitral, and geographic coordinates as noted by GPS were N 36°22.616 and E 072°08.983. The elevation of the site was 10,941 feet. In the current study, total 13 bacterial strains have been isolated from surface ice, water and sediments of Tirich Mir glacier, Chitral but only 3 isolates were proceeded further. The highest viable cell count was found in surface ice that was 2.3×10^5 CFU/gm followed by water and sediments having 1.7×10^3 and 1.3×10^3 CFU/mL,mg respectively.

Table 1. Morphological and microscopic characteristics of bacteria isolates.

Isolates	Morphological characteristics	Microscopic characteristics
TG1-MRL	Large, circular shaped with off-white color, opaque opacity with entire margin	Gram positive, long filamentous chains, rods
TG3-MRL	Medium , circular shaped with white color then turn Brick red, opaque opacity with entire margin	Gram negative, short rods , Scattered
TG4-MRL	Medium , circular shaped with orange color, opaque opacity with entire margin	Gram positive, rods, Short chains

Characterization of Isolates

The bacterial isolates were of different color, medium to large size with opaque opacity. Gram's staining of the isolates revealed that two isolates TG1-MRL and TG4-MRLwere Gram positive while one isolate TG3-MRLwas Gram negative. Colony morphology and microscopy of all isolates is shown in Table 1.The response of bacterial isolates was varied to biochemical tests performed conventionally(Table 2).

Isolates TG1-MRL and TG3-MRL showed growth up to 35°C while TG4-MRL grown up to 25°C. The isolates responded differently to salts concentrations. TG3-MRL demonstrated at 5% NaCl concentration while TG4-MRL was able to grow at 9% NaCl. In addition, the pH tolerance of two isolates was observed between pH 3-11 but TG1-MRL grown at pH range 5-7. The growth of bacterial isolates at different temperatures, salts concentrations and pH is given in Table 2.

Table 2. Physiological and biochemical analysis of the bacterial isolates.

S.No	Characteristic	TG1-MRL	TG3-MRL	TG4-MRL
1	Physiological analysis			
	Temperature °C	15-35	5-35	15-25
	pH	5-7	3-11	3-11
	Salt concentrations (%)	1-3	1-5	1-9
2	Biochemical analysis			
	Glucose	+	+	+
	Lactose	-	+	+
	Sucrose	-	+	+
	Oxidase test	-	-	-
	Nitrate reduction	+	+	-
	Catalase test	+	+	+
	TSI			
	Slant	K	A	A
	Butt	K	A	A
	Gas	-	-	-
	H_2S production	-	-	-
Legend	(+) Positive, (-) Negative, (A) Acid production, (K) alkaline reaction			

Based on 16S rRNA sequencing, isolates belonged to two major groups. The phylogenetic relationship of isolate to different related bacterial species is given Figure 1. It was found that isolate TG1-MRL, TG3-MRL and TG4-MRL showed 99% similarity to *Bacillus cereus*, *Serratia* sp. and *E. sibiricum* respectively after BLAST search in NCBI.

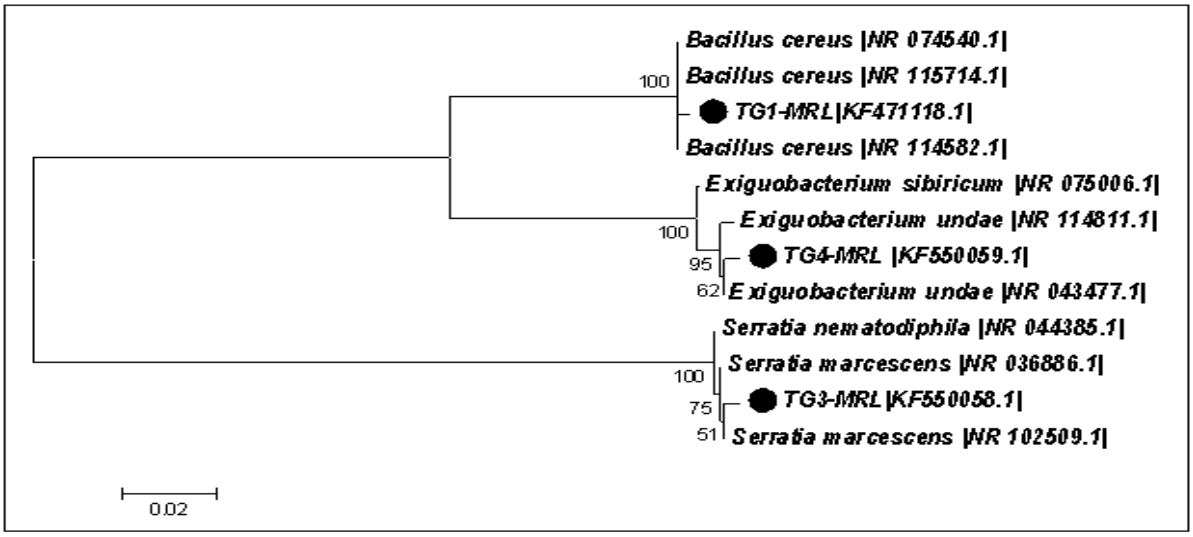

Fig. 1.

Protease production assay

All isolates were able to produce protease but the best protease producers were TG3-MRL and TG4-MRL as compared to TG1-MRL (Fig. 2).For protease assay, the tyrosine (µg/mL) was calculated. The standard curve was plotted using the trend line equation y = 0.0861x - 0.0513. The optimum protease production for TG3-MRL was observed after 4 days of incubation at 15°C with specific activity of 4.40 U/mg. The lag phase of TG1-MRL was achieved after 3-4 days while maximum specific activity of 3.69 U/mg was attained after 96 hours. The specific activity declined until a straight curve was obtained after 144 hours up to 196 hours. TG4-MRL log phase observed after 96 hours of incubation at 15°C with specific activity 2.42 U/mg. After 120 hours it entered its decline phase. The relationship between growth and specific activity (U/mg) of all isolates is shown in Fig. 3.

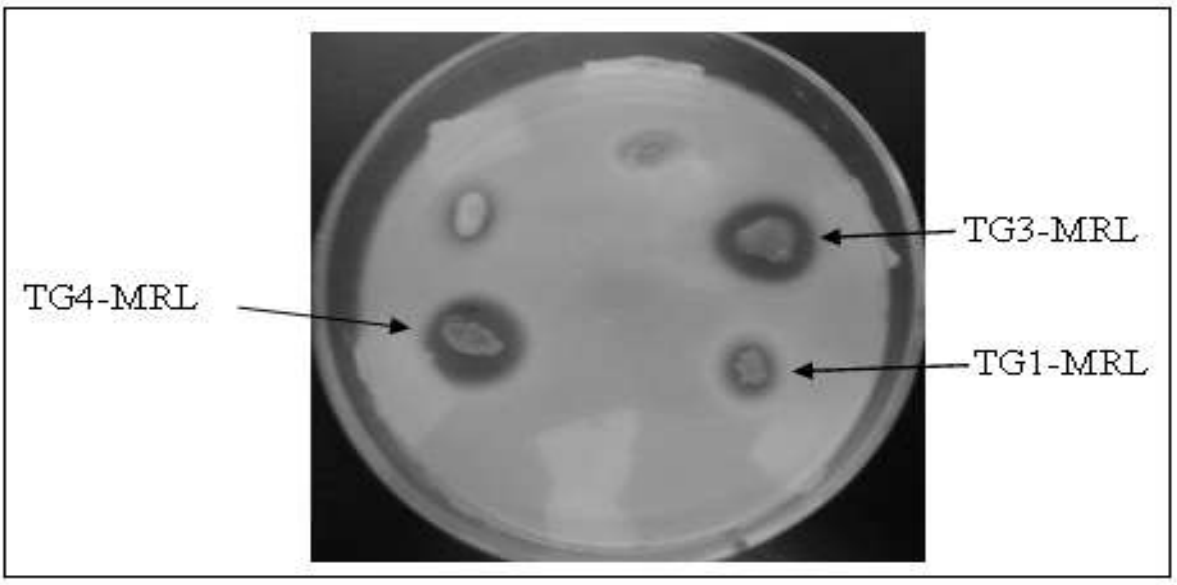

Fig. 2.

Characterization of Crude Extracellular Protease Extracts

Effect of pH

The optimum pH range for proteases production was 7-9 for all strains. Proteases from TG1-MRL and TG4-MRL was stable at broad range of pH (3-11), while the protease from TG3-MRL showed decrease in residual activity at acidic pH and 50% of activity was lost at pH 3 (Fig. 4a).

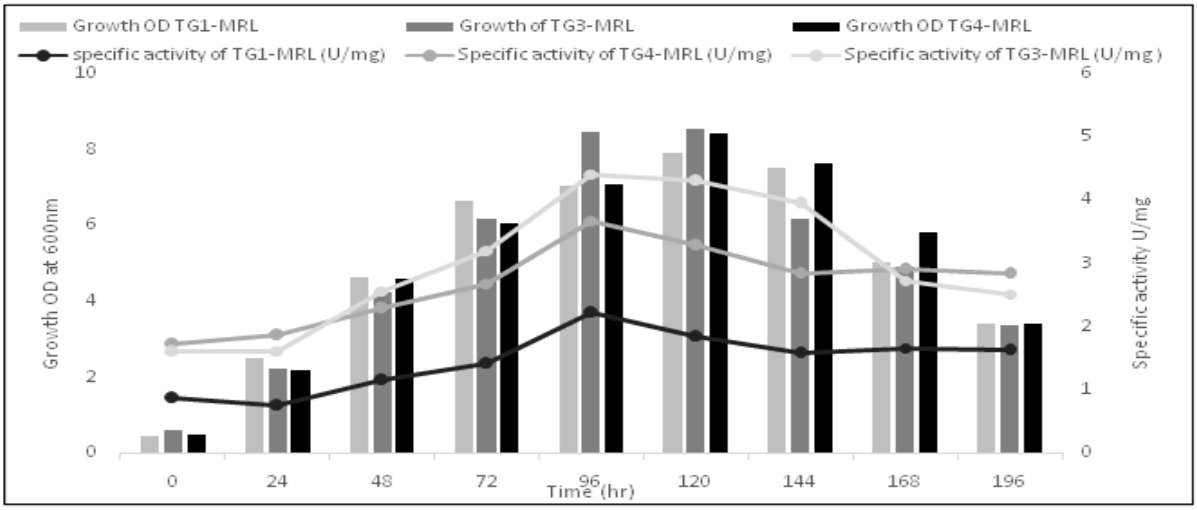

Fig. 3.

Effect of temperature

The protease stability of TG1-MRL was observed at temperature ranges from 5- 55°C, with residual activity of 95-99%, but lost 33% of its activity at 65°C.The protease from TG3-MRL was sensitive to high temperature and showed more activity at low temperature ranging from 99-110% residual activity at 5-45°C. Protease produced by TG4-MRL showed maximum stability with residual activity of 117% at 35°C. Its activity decreased gradually from 35 to 65°C. It retained almost 60% of its activity at 65°C (Fig. 4b).

Effect of Divalent Metal Ions

An increase in the stability of protease production was observed in the presence of metal ions by all the three strains. The protease activity from TG1-MRL increased by 10% in the presence of Ca^{+2}. Whereas, more than 90% activity was retained by this enzyme in the presence of Na^{+}, Hg^{+2}, Mg^{+2} ions, 26% of activity was lost when incubated with 10 mM Zn^{+2} for 3 hours at 15°C. TG3-MRL produced protease with the stable and increased residual activity from 111-129% in the presence of Na^{+}, Hg^{+2} and Mg^{+2}. While 92% of the activity was achieved in the presence of Ca^{+2} but its activity reduced to less than 60% on treatment with Zn^{+2}. The protease from TG4-MRL showed its residual activity increased from 103-112% in the presence of Hg^{+2} and Ca^{+2}. Less than 100% activity was achieved when treated in the presence of Na^{+} and Mg^{+2} (93.37% and 97.79%), whereas, 17% of the protease activity was lost after 3 hours of incubation with 10 mM Zn^{+2}(Fig. 4c).

Effect of modulators and inhibitors on the activity of crude enzyme extracts

In the presence of 1% mercaptoethanol, the residual activity of protease produced by TG1-MRL and TG3-MRL, retained up to 80%, while 25% of activity was lost by protease produced by TG4-MRL.Proteases from TG1-MRL and TG3-MRL, retained 90% of activity in presence of EDTA, whereas, protease from TG4-MRL showed destabilization and lost 30% of its activity. In the presence of 1% PMSF, proteases from TG1-MRL and TG3-MRL showed stability and nearly 92% of its activity was maintained. In contrast, protease from TG4-MRL showed significant reduction and only 46% of activity was exhibited by this enzyme. Its activity increased up to 127% in the presence of phenyl acetaldehyde and not affected by trisodium citrate. A decreased residual activity was

shown by protease produced by TG1-MRL in the presence of phenyl acetaldehyde (20.85%) and trisodium citrate (72.34%), while activity of protease from TG3-MRL was not effected by phenyl acetaldehyde and increased up to 105% in case of trisodium citrate (Fig. 4d).

Discussion

The main theme of our study was isolation and characterization of extracellular protease from the cold tolerant bacteria isolated from samples of glacial ice, sediment and melted water, taken from Tirich Mir glacier, Chitral, Pakistan. A total 13 bacterial strains were isolated and only 3 isolates, namely TG1-MRL, TG3-MRL and TG4-MRL, were subjected for the further research work. These bacteria were identified as *Bacilluscereus, Serratia* sp. and *Exiguobacterium* sp. TG1-MRL, TG3-MRL and TG4-MRL, all showed 99% similarity with *Bacilluscereus, Serratia* sp. and *Exiguobacterium* sp., respectively. Isolation of cold tolerant protease-producing bacteria belonging to those genera has been earlier reported from various cold environments (Dancer *et al.,* 1997; Miteva *et al.,* 2004; Qiu *et al.,* 2006; Rodrigues *et al.,* 2008; Ahmad *et al.,* 2010; Tariq *et al.,* 2011).

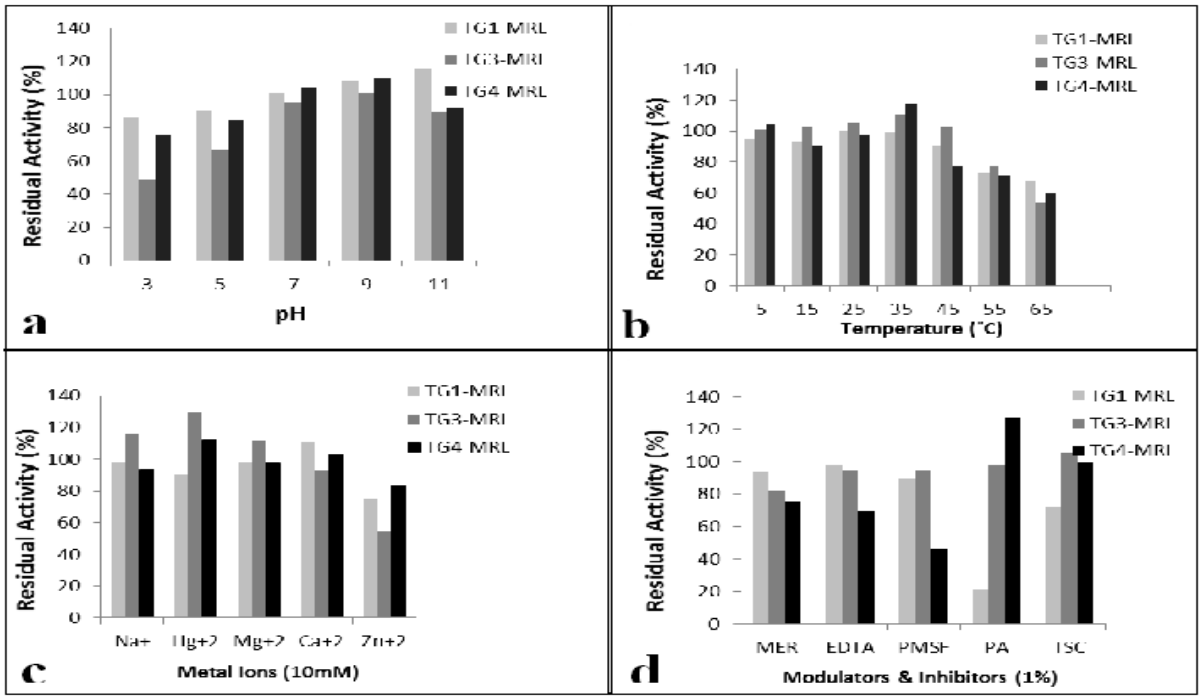

Fig. 4.

In this study, extracellular crude protease was isolated and comparatively characterized with respect to their quantitative and stability aspects, from the three bacteria isolates (TG1-MRL, TG3-MRL and TG4-MRL). The maximum proteolytic activity of isolates TG1-MRL was 3.69 U/mg, TG3-MRL was 4.40 U/mg and TG4-MRL was 2.42 U/mg that was recorded after incubation time of 96 hours. The protease production from psychrotrophs during late log, early and late stationary phase has been observed in earlier studies (Vazqueza *et al.,* 2004; Chauhan and Gupta, 2004; Huston *et al.,* 2004; Shama and Hameed, 2011).

The effect of physiological parameters on the production of extracellular proteolytic enzymes could play an important role in the induction or repression of the enzyme. In this research work, proteases have showed a wide range of stability at different pH and temperatures. The protease stability from all the isolates was observed at temperature ranges from 5-55°C but was maximum at 35°C. However, Protease from TG3-MRLand TG1-MRL, destabilized with increase in temperature and lost its activity up to 50%

and 33% at 65°C, respectively. Cold active enzymes with optimum activity at 35-45°C, has been reported from the cold adapted bacteria (Doddapaneni *et al.*, 2007; Cotarlet *et al.*, 2009). However, Margesin *et al.* (2005) has isolated cold active enzymes with an optimum activity even at 60°C. The maximum proteolytic activity of all the isolates exhibited pH 7-9. TG1-MRLretained 90% of the residual activity at pH 5.0 and 3.0 as well. However, Protease from TG3-MRL, lost 50% of activity at pH 3. Doddapaneni *et al.* (2007) and Rao *et al.* (2008) reported cold adapted bacteria that are able to produce hydrolytic enzymes with narrow range of pH stability. Hamamoto *et al.* (1994) isolated extracellular proteases from a psychrotrophic *Pseudomonas fluorescens* with optimum protease activity between pH 6.5 and 10.

The effects of different divalent metal ions on the extracellular crude proteases, was investigated in this study. The residual activity of proteases from TG1-MRL, TG3-MRL and TG4-MRL was observed stable to Na^{+}, Mg^{+2}, Ca^{+2} and even to Hg^{+2} which possess inhibitory effects in more cases. The proteolytic activity of protease from TG3-MRLwas increased by Na^{+2}, Hg^{+2} and Mg^{+2} up to 15, 29 and 11% respectively. 46% of its activity was lost upon treatment with Zn^{+2}, while K^{+} did not affect the protease. Protease fromTG4-MRLshowed stability in the presence of Zn^{+2}. Same results were reported by various authors (Zeng *et al.*, 2003;Vazqueza *et al.*, 2004; Kasana and Yadav, 2007) was reported the negative effects of Hg^{+2}, Zn^{+2}, Cu^{+2} on the proteases stability produced by psychrotolerant *Pseudomonas* sp.

In this research work, influences of different modulators and inhibitors on the extracellular crude proteases were studied. EDTA is a chelating agent that may binds to the metal ions present in the active site of enzymes, to bind the substrate but EDTA showed a negligible effect on all the proteases produced by the three isolates. This provides evidence that the proteases are nonmetallic in nature. Rao *et al.* (2008) also reported alkane proteases that were not destabilized by EDTA. Phenylmethyl sulfonylfluoride (PMSF) inhibits the binding of enzyme to its substrate by adding sulfonyl group to the serine residues (Adinarayana *et al.*, 2003). Proteases from strains TG1-MRL and TG3-MRL showed significant stability even at high concentration of PMSF (100 mM), while 54% of activity was lost by proteases from TG4-MRLwhich provided a clue that this protease might be serine protease. A similar result was reported from protease producing *Pseudomonas* species isolated from cold environment (Zeng *et al.*, 2003). Mercaptoethanol can affect the enzyme by blocking the histidine and disulfide bonds, which are required for the enzyme substrate reaction. In our studies, the residual activity of proteases from all the isolates was retained up to 90% to mercaptoethanol. Margesin *et al.* (2002) reported protease from *Serratia marcescens* that retained 83% activity to mercaptoethanol.

Conclusion

Three potent protease producing bacteria were isolated and characterized from Tirich Mir glaciers. The culture conditions and media components were improved for better production of proteases. The studied proteases from psychrophilic bacteria were neutral, with reduced thermal stability, but active at a reasonably comprehensive range of temperature, metal ions and pH. These properties make them possibly useful for industrial applications on practices carried out at neutral pH and ambient temperature in temperate-climate regions, as these enzymes have their optimal activity at temperatures where the mesophilic enzymes show a significant decline of their utmost activity. Such processes should be done at temperatures below 45°C and between pH 5 and 10 to pledge the solidity of the proteases along the process. Further experiments will be carried out to obtain high yield of protease for purification and characterization for industrial use.

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