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Purification and characterization of protease enzyme from actinomycetes and its cytotoxic effect on cancer cell line (A549)

C Balachandran, V Duraipandiyan, S Ignacimuthu*

Division of Microbiology, Entomology Research Institute, Loyola College, Chennai-600034, India

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ABSTRACT

Objective: To isolate active actinomycetes from soil samples of Northern Himalayas and study their culture characterization, protease production and cytotoxic effects on cancer cell line (A549). **Methods:** Forty six strains of actinomycetes were isolated from the soil collected from Northern Himalayas, India. Isolation of actinomycetes was performed by serial dilution plate technique. Forty six isolated actinomycetes cultures were grown in ISP 2 medium to study the morphology and biochemical characteristics. Isolated strains were studied for protease enzyme production in skim milk agar medium with solubilising capacity. Seven isolates were studied for melanin pigmentation and different NaCl concentration. Effects of environmental conditions influencing protease enzyme production of seven isolated strains were also studied at different pH, temperature and metal ions (β-mercaptoethanol, dithiothreitol, iodoacetamide, $MgSO_4$, $CaCl_2$ and EDTA). The seven isolates were also studied for lytic enzyme activity using different bacteria and yeast such as *Pseudomonas aeruginosa (P. aeruginosa), Enterococcus feacalis (E. feacalis), Escherishia coli (E. coli), Candida albicans (C. albicans), Bacillus subtilis (B. subtilis), Klebsiella pneumonia (K. pneumonia)* and *Staphylococcus aureus (S. aureus)*. **Results:** Isolates ERIA-31 and ERIA-33 produced more protease enzyme activity in modified nutrient agar media compared to other actinomycetes cultures. ERIA-31 and ERIA-33 were tested for cytotoxic effect in human adenocarcinoma cancer cell line (A549). IC_{50} for ERIA-31 was 57.04 μg/mL and IC_{50} for ERIA-33 was 55.07 μg/mL. **Conclusion:** Actinomycete being a protease producing bacteria has the potential for use in industrial purpose, pharmaceuticals, cytotoxic agent and its proteolytic activity. Isolates of ERIA-31 and ERIA-33 produced significant amount of protease enzymes.

1. Introduction

Proteases are the most important group of the enzymes produced commercially and industrial purpose[1,2]. They have extensive applications in a range of industrial products and processes including detergents, food, pharmaceuticals and leather[3,4]. They are classified into various groups such as alkaline protease, serine protease, cystein protease, aspartic protease and metallo protease[5]. Protease constitutes one of the most important groups of industrial enzymes, accounting for at least 25% of the total enzyme sales, with two-thirds of the proteases produced commercially being of microbial origin.

In recent years a number of studies have been conducted to characterize protease from different microorganisms. The production of proteases by microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, and by physical factors such as temperature, pH, incubation time, agitation and inoculum density. Although protease production is an inherent property of all organisms, only those microbes that produce a substantial amount of extracellular protease have been exploited commercially. They also include species that are able to degrade many macromolecules such as lipids, starch, chitin, pectin, and proteins[6]. While proteases from bacteria are extensively characterized, similar attention has not been paid to actinomycetes.

Actinomycetes are Gram-positive, mycelium-forming soil bacteria that include many species considered to be among the most important producers of antibiotics[7].

*Corresponding author: Dr. S Ignacimuthu, Director, Entomology Research Institute, Loyola College, Nungambakkam, Chennai - 600 034, Tamil Nadu, India.
Tel: 044 2817 8348
Fax: 044 2817 5566
E-mail: entolc@hotmail.com
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However, the present knowledge concerning proteases of actinomycetes is much less than that of fungi and other bacteria. Proteases produced by actinomycetes are the most important group of secondary metabolites that are widely exploited. Further proteases from *Streptomyces* sp.[8], *Bacillus* Sp.[2,9-11] *Myceliophthora* sp.[12], *Aspergillus fumigates*, *Aspergillus awamori* and *Aspergillus niger*[13-15] were reported. Most of the studies on actinomycetes have focused on antibiotic production; only few reports are available on their enzymatic potential[16]. The present study was aimed at the purification of protease enzyme from actinomycetes isolated from Northern Himalayas, India and assessing its cytotoxic effect on cancer cell line (A549).

2. Materials and methods

2.1. Sample collection

The soil samples were collected at the depth of 5-15 cm from Northern Himalayas, India.

2.2. Isolation of actinomycetes

The soil samples were air dried at 100 ℃ for 1 h and then ground in a mortar using pestle. Isolation of actinomycetes was performed by serial dilution using dilution plate technique. One gram of soil was suspended in 9 mL of sterile distilled water. The dilution was carried out up to 10^{-6} dilutions. Aliquots (0.1 mL) of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were spread on the isolation plates containing starch casein agar (SCA)[17], and actinomycetes isolation agar (AIA) (Himedia, Mumbai). To minimize the bacterial and fungal growth, actidione 30 mg/L and nalidixic acids 40 mg/L were added. The plates were incubated at 28 ℃ for 7 to 21 d. The isolates were grown on ISP-2 agar medium at 28 ℃ and stored at 4 ℃ for short term storage. The isolates were grown in MNGA broth for 5 d; then 20% of glycerol and 80% of sample were mixed and stored at -20 ℃ for long term storage.

2.3. Morphological characteristics

Actinomycetes colonies were characterized morphologically and physiologically following the direction given by the International Streptomyces project (ISP) and Bergey's manual of Systematic Bacteriology. Cultural characteristics of pure isolates after incubation in different media via ISP-2, ISP-3 and ISP-4 were recorded for 7 and 14 d at 28 ℃. Morphological observations were made with light microscope using the method of Buchnan and Gibbons[18]. Actinomycetes were identified to the species level by comparing the morphology of spore bearing hyphae with entire spore chain and structure of spore as described in Bergey's manual and Actinomycetes manual. Physiological and biochemical characteristics of the isolates cultured on ISP-6 media were examined for melanin synthesis. Gram staining method of Duraipandiyan *et al*[19] was followed. Proteolytic activity of isolated actinomycetes was screened by its solubilsing capacity on Skim milk agar with the following composition in g per litter: 3 g of Yeast extract, 5 g of NaCl, 10 g of Skim milk, 18 g of Agar and pH 7.2. Solubilising capacity was calculated using the following formula: Solubilising capacity=Solubilising diameter/organism diameter×100.

2.4. Carbohydrate fermentation test

The carbohydrate fermentation tests for actinomycetes isolated from Himalayan soil sample were carried out according to the method of Kandler and Weiss[20]. Nine carbohydrate sugar substrates *viz.*, dextrose, galactose, mannose, ribose, rhamnose, xylose, arabinose, sucrose and manitol were used. Carbohydrate fermentation patterns of the isolates were compared with Bergey's manual of Systematic Bacteriology.

2.5. Protease assay

The protease activity was determined with casein as a substrate following the procedure of Abidi *et al*[21]. The reaction mixture contained 50 μL of Tris-HCl buffer (pH 8.5), 2 mg/mL of casein, and 50 μL of enzyme solution. The mixture was incubated at 30 ℃ for 25 min and the reaction was stopped by the addition of an equal volume of 10% trichloroacetic acid (TCA). After centrifugation, absorbance of supernatant was read at 280 nm. One unit of proteolytic activity was defined as the amount of enzyme which liberates digestion product not precipitated by TCA equivalent to 1 μmol of tyrosine per minute under the assay conditions.

2.6. Effect of metal ions

The effect of different metal ions ($MgSO_4$, $CaCl_2$ and EDTA) on enzyme activity was studied by incubating the enzyme with a 10 mM concentration of different metal ions for 1 h. The enzyme activity calculated without metal ions and inhibitor were used as control. Furthermore, the effect of various compounds on enzyme activity was checked by incubating the enzyme with a 10 mM concentration of different compounds *viz.*, β-mercaptoethanol, dithiothreitol and iodoacetamide for 1 h. The relative activities were estimated with reference to control[22].

2.7. Effects of environmental conditions

To check the effect of pH on the activity of enzyme, reaction mixture with enzyme was incubated for 25 min

at 30 ℃ in 50 μL of sodium acetate buffer at pH 3-5 and 50 μL of Tris-HCl buffer at pH 4-9 using 2 mg/mL of casein as a substrate. The effect of temperature on the stability was studied by incubating the enzyme at different temperatures (4, 10, 28, 37 and 50 ℃) for 1 h. The activity obtained at 37 ℃ was used as control[21].

2.8. Protease enzyme production in liquid culture

The inoculum was prepared by transferring a loop full of culture from the slant into 25 mL sterile modified nutrient glucose agar (MNGA) medium containing g/L, 10 g of glucose, 5 g of peptone, 3 g of beef extract, 3 g of dry yeast extract, 3 g of NaCl and 3 g of $CaCO_3$ followed by incubation at 30 ℃ on a rotary shaker (200 rpm) for 3 d. Ten percent of this activated culture was inoculated in 100 mL of casein broth (5% w/v NaCl, pH 9) and incubated at 30 ℃ under shaking conditions as described above. After 3rd day cells were harvested by filtering the broth with cellulose filter; subsequently, 1 mL of this culture was transferred to 100 mL of casein broth and incubated under the same conditions for 12 h. The culture was centrifuged at 12 000 rpm for 25 min at 4 ℃ and the supernatant was collected in fresh flask. The filtrate was used as the crude enzyme[22]. The purified enzyme was using for further studies.

2.9. Bacteriolytic activity

The lytic enzyme activity was determined by adding 50 mL of test sample to 220 mL of bacterial suspension (200 mg^{-1} in 0.01 m phosphate buffer, pH 6.2 containing 500 mg^{-1} bovine serum albumin and 2 mm DTT) in plastic 96 well plates. The optical density at 450 nm was determined at the beginning of the assay and the plate was incubated at 37 ℃. The lytic enzyme activity was measured by decrease in absorbance value. *Pseudomonas aerougenosa* (*P. aerougenosa*) MTCC 741, *Enterococcus feacalis* (*E. feacalis*) ATCC 29212, *Escherishia coli* (*E. coli*) ATCC 25922, *Bacillus subtillus* (*B. subtillus*) MTCC 441, *Klebsiella pneumonia* (*K. pneumonia*) MTCC 109, *Staphylococcus aureus (S. aureus*) MTCC 96 and *Candida albicans* (*C. albicans*) MTCC 227 were used for this assay[23].

2.10. Determination of cytotoxicity effect on A549 cancer cell

A549 human adenocarcinoma cell line was received from National Institute of Cell Sciences, Pune. A549 cell line was maintained in complete tissue culture medium DMEM with 10% fetal Bovine serum and 2 mM L-Glutamine, along with antibiotics (about 100 IU/mL of penicillin, 100 μg/mL of streptomycin) with the pH adjusted to 7.2. The cytotoxicity was determined according to the method of Hsu *et al*[24] with some changes. Cells (5×10^4) were seeded in 96 well plates containing medium with different concentrations such as enzyme I (ERIA-31) 30 μg, 15 μg, 7.5 μg, enzyme II (ERIA-33) 20 μg, 10 μg, 5 μg and a set as control for a 24 h study. The cells were cultivated at 37 ℃ with 5% CO_2 and 95% air in 100% relative humidity. After various durations of cultivation, the medium solution was removed. An aliquot of 100 μL of medium containing 1 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was loaded to the plate. The cells were cultured for 4 h and then the medium solution was removed. An aliquot of 100 μL of DMSO was added to the plate, which was shaken until the crystals were dissolved. The cytotoxicity against cancer cells was determined by measuring the absorbance of the converted dye at 570 nm in an ELISA reader. Cytotoxicity of each sample is expressed as IC_{50} value. The IC_{50} value is the concentration of test sample that causes 50% inhibition of cell growth, averaged from three replicate experiments, which is obtained by plotting the percentage inhibition versus concentration of test sample. Growth inhibition was calculated from the following formula: (% Cytotoxicity) = {[1-(A/B)]×100}. 5-FU was used as the positive control, an effective anticancer drug.

3. Results

Forty six actinomycetes were isolated from the Northern Himalayas. The pure colonies were designated as ERIA-1 to ERIA-46 (Entomology Research Institute Actinomycetes). The forty six isolates were maintained in the ISP-2 slant at 4 ℃. Morphology of the actinomycetes colonies was determined in the selective media from which they had been isolated. Isolates showed pigmentation, filamentous colony margin and produced mycelium, aerial hyphae and spores (Table 1) (Figure 1). Proteolytic activity of isolated actinomycetes on Skim milk agar medium was screened by its solubilsing capacity. More than seven isolates produced protease enzyme in skim milk agar media (ERIA - 3, 7, 22, 27, 31, 33 and 40) (Table 2). These seven isolates were tested for melanin pigment production on peptone yeast extract iron agar (ISP-6) (Table 3). The ability of actinomycetes to grow at various concentration of NaCl in the media was thoroughly studied. All the isolates grew well at 1.5%, 3% and 4.5% of NaCl. There was no growth at 5% to 15% concentrations of NaCl (Table 4). Carbohydrate fermentation tests were carried out for isolated actinomycetes cultures. The strains showed similarity in their ability to assimilate nine carbon sources. However, some differences occurred in the use of dextrose, mannose, ribose, sucrose and mannitol. The strains differed among themselves in the ability to produce acid. The seven isolates *viz*., ERIA- 3, 7, 22, 31, 33, and 40 fermented in dextrose and ribose significantly (Table 5). All metal ions used in this study showed an

Table 1.
Morphological characteristics of isolated actinomycetes.

Isolates	Growth ISP-2	Aerial mycelium colour	Substrate mycelium colour	Soluble pigment colour	Colony margin	Colony elevation	Gram staining
ERIA- 1	+	White sandal	Yellow -red	No	Filamentous	Flat	+
ERIA- 2	+	Brown-gray	Red-brown	weak yellow	Filamentous	Flat	+
ERIA- 3	+	Dark gray	Golden yellow	Yellow	Filamentous	convex	+
ERIA- 4	+	Whitish gray	Light chocolate	No	Filamentous	Irregular	+
ERIA- 5	+	White	Yellow brown	No	Filamentous	Flat apex	+
ERIA-6	+	Sandal yellow	Light brown	No	Filamentous	Flat apex	+
ERIA- 7	+	Whitish gray	Greenish black	No	Filamentous	Flat	+
ERIA- 8	+	Black yellow	Golden yellow	Light	Filamentous	Convex	+
ERIA- 9	+	White	Golden yellow	No	Filamentous	Flat apex	+
ERIA- 10	+	White yellow	Sandal yellow	No	Filamentous	convex	+
ERIA- 11	+	White gray	Chocolate	Light brown	Filamentous	convex	+
ERIA- 12	+	Yellow	Yellow	No	Filamentous	Convex	+
ERIA- 13	+	Orange	Chocolate Red	Light orange	Filamentous	Flat apex	+
ERIA- 14	+	Milky white	White	No	Filamentous	flat	+
ERIA- 15	+	White	White red	Yellow red	Filamentous	Apex	+
ERIA- 16	+	White gray	Black	No	Filamentous	Flat	+
ERIA- 17	+	White	Light yellow	No	Filamentous	Flat apex	+
ERIA- 18	+	White	White	No	Filamentous	Flat	+
ERIA- 19	+	Gray	Light yellow	No	Filamentous	Flat	+
ERIA- 20	+	Yellowish gray	Yellowish gray	No	Filamentous	Convex	+
ERIA- 21	+	Milky white	Yellow gray	No	Filamentous	Flat apex	+
ERIA- 22	+	White	Grayish white	No	Filamentous	Flat	+
ERIA- 23	+	Gray white	Gray white	No	Filamentous	Flat apex	+
ERIA- 24	+	Light brown	Light yellow	No	Filamentous	Flat	+
ERIA- 25	+	White	Brown	Light yellow	Filamentous	Apex	+
ERIA- 26	+	White	Brown	No	Filamentous	Flat apex	+
ERIA- 27	+	Gray white	Brown	Pale pink	Filamentous	Flat apex	+
ERIA- 28	+	White	White	-	Filamentous	Flat	+
ERIA - 29	+	White	White	No	Filamentous	Apex	+
ERIA- 30	+	White	White	No	Filamentous	Flat	+
ERIA- 31	+	Light yellow	Pale yellow	No	Filamentous	Flat apex	+
ERIA- 32	+	Whitish gray	Dark brown	Pale brown	Filamentous	convex	+
ERIA - 33	+	Light yellow	Golden brown	No	Filamentous	Flat	+
ERIA - 34	+	Pinky white	Chocolate grey	No	Filamentous	Flat apex	+
ERIA - 35	+	Sandal white	Golden yellow	No	Filamentous	Apex	+
ERIA - 36	+	White	Golden yellow	No	Filamentous	Apex	+
ERIA - 37	+	Yellow	Yellow	No	Filamentous	Flat	+
ERIA - 38	+	Gray white	Chocolate	No	Filamentous	Flat	+
ERIA - 39	+	Yellow	Yellow	No	Filamentous	Flat	+
ERIA - 40	+	White	White	No	Filamentous	Flat	+
ERIA - 41	+	White	White	No	Filamentous	Flat	+
ERIA - 42	+	White	White	No	Filamentous	Flat	+
ERIA - 43	+	White	White	No	Filamentous	Flat	+
ERIA - 44	+	White	White	No	Filamentous	Flat	+
ERIA - 45	+	Brownish gray	Golden yellow	No	Filamentous	Flat	+
ERIA - 46	+	White	White	No	Filamentous	Flat	+

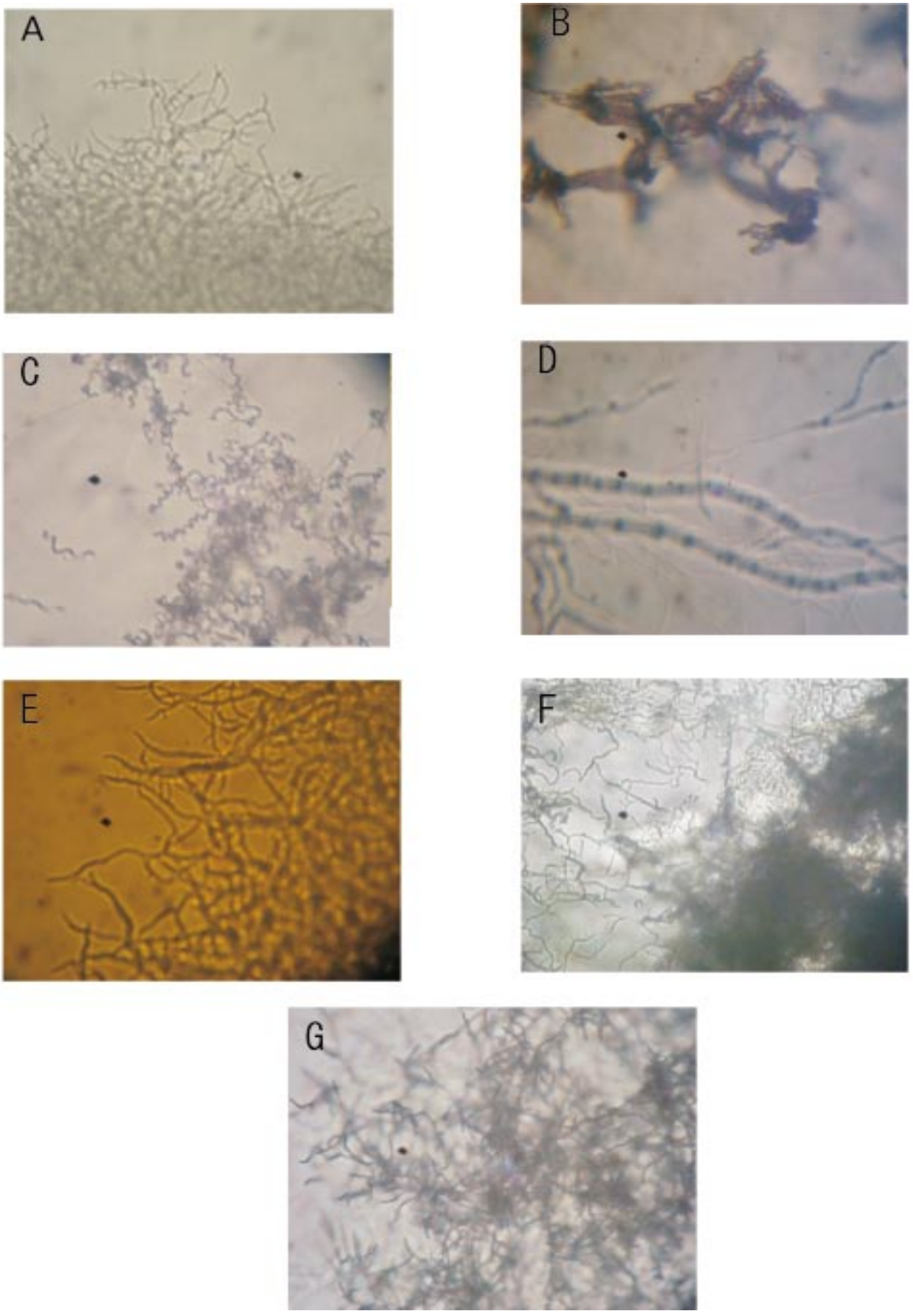

Figure 1. Microscopic analysis A.ERIA-3,B.ERIA-7,C.ERIA-22,D.ERIA-27,E.ERIA-31 and G.ERIA-40.

Table 2.
Screening of proteolytic activity of isolated actinomycetes on skim milk agar media.

Isolates	+ ve	− ve	Solubilising capacity
ERIA− 1	−	+	200.00
ERIA− 2	+	−	150.00
ERIA− 3	+++	−	350.00
ERIA− 4	−	+	200.00
ERIA− 5	−	+	120.00
ERIA− 6	+	−	200.00
ERIA− 7	+++	−	300.00
ERIA− 8	+	−	200.00
ERIA− 9	++	−	233.33
ERIA− 10	−	+	200.00
ERIA− 11	++	−	216.67
ERIA− 12	−	+	200.00
ERIA− 13	−	+	200.00
ERIA− 14	+	−	133.33
ERIA− 15	−	+	200.00
ERIA− 16	+	−	200.00
ERIA− 17	++	−	166.67
ERIA− 18	++	−	225.00
ERIA− 19	++	−	222.22
ERIA− 20	−	−	200.00
ERIA− 21	++	−	260.00
ERIA− 22	+++	−	300.00
ERIA− 23	−	+	120.00
ERIA− 24	−	+	120.00
ERIA− 25	−	+	120.00
ERIA− 26	++	−	120.00
ERIA− 27	+++	−	300.00
ERIA −28	−	+	120.00
ERIA− 29	+	−	140.00
ERIA − 30	−	+	120.00
ERIA− 31	+++	−	400.00
ERIA− 32	−	+	120.00
ERIA− 33	+++	−	387.50
ERIA − 34	−	+	140.00
ERIA − 35	−	+	120.00
ERIA − 36	++	−	200.00
ERIA − 37	−	+	120.00
ERIA − 38	−	+	120.00
ERIA − 39	++	−	250.00
ERIA − 40	+++	−	305.45
ERIA − 41	++	−	236.36
ERIA − 42	++	−	207.69
ERIA − 43	++	−	216.45
ERIA − 44	+	−	120.00
ERIA − 45	++	−	223.08
ERIA − 46	−	+	120.00

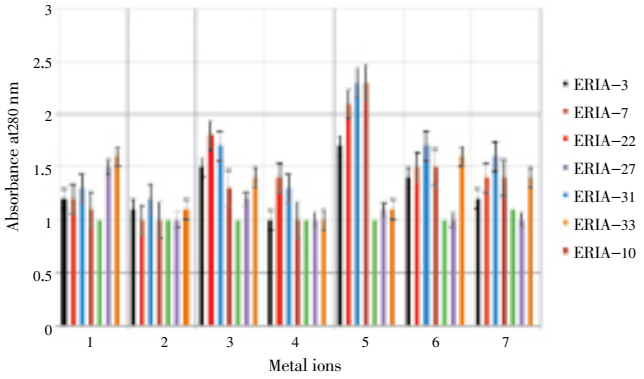

Figure 2. Metal ions.
1. Control; 2. Iodoacetamide; 3. β −mercaproethanol; 4. Dithiothreitol; 5. $MgSO_3$; 6. $CaCl_2$; 7. EDTA.

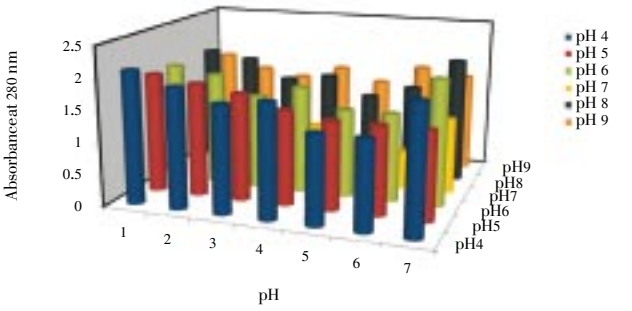

Figure 3. Effect of pH (1. ERIA−3; 2. ERIA−7; 3. ERIA−22; 4. ERIA−27; 5. ERIA−31; 6. ERIA−33 and 7. ERIA−40).

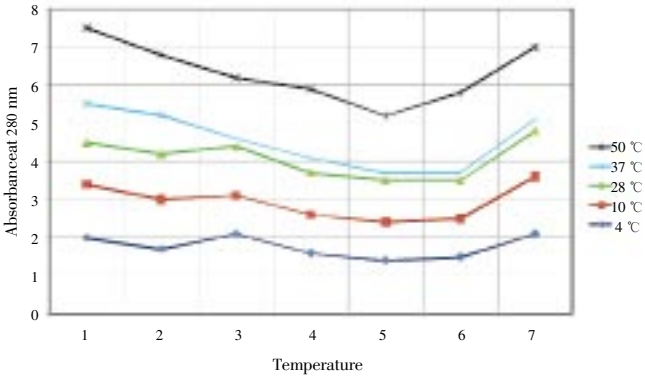

Figure 4. Effectf temperature (1. ERIA−3; 2. ERIA−7; 3. ERIA−22, 4. ERIA−27; 5. ERIA−31; 6. ERIA−33 and 7. ERIA−40).

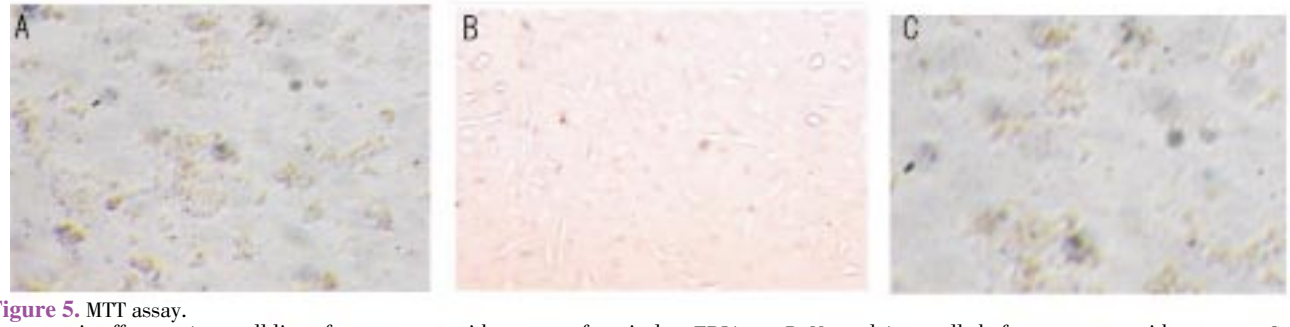

Figure 5. MTT assay.
A. cytotoxic effect on A549 cell line after treatment with protease from isolate ERIA−31; B. Normal A549 cells before treatment with protease; C. Cytotoxic effect on A549 cell line after treatment with protease from isolate ERIA−31.

enhancing effect on the activity of the enzyme. However, β – mercaptoethanol, dithiothreitol, and EDTA at 10 mM concentration had no effect on the activity of the enzyme.

Table 3.
Melanin pigmentation.

Isolates	+ve	-ve
ERIA – 3	+	–
ERIA – 7	–	+
ERIA – 22	+	–
ERIA – 27	–	+
ERIA – 31	++	–
ERIA – 33	++	–
ERIA – 40	+	–

– no growth ; + moderate growth; ++ good growth.

In the presence of 10 mM $CaCl_2$ ion, enzymatic activity increased. Similar activity was also observed in $MgSO_4$. However, in the presence of the $MgSO_4$ ions, the enzyme

Table 4.
Utilizing of NaCl (Different concentration).

Isolates	1.5%	3.0%	4.5%	6.0%	9.5%	12.0%	15.0%
ERIA – 3	+	+	–	–	–	–	–
ERIA – 7	++	+	+	–	–	–	–
ERIA – 22	++	++	+	–	–	–	–
ERIA – 27	+	+	+	–	–	–	–
ERIA – 31	++	++	++	–	–	–	–
ERIA – 33	++	++	++	–	–	–	–
ERIA – 40	+	+	+	–	–	–	–

– no growth; + moderate growth; ++ good growth.

Table 5.
Carbohydrate fermentation test.

Isolates	Dexstrose		Galactose		Mannose		Ribose		Rhamnose		xylose		Arabinose		sucrose		Mannitol	
	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
ERIA – 3	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
ERIA – 7	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
ERIA – 22	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
ERIA – 27	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
ERIA – 31	+	–	–	–	+	–	+	–	–	–	–	–	–	+	+	–	–	–
ERIA – 33	+	–	–	–	+	–	+	–	–	–	+	–	–	–	+	–	+	–
ERIA – 40	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–

+ Active culture – no growth observed.

Table 6.
Bacteriolytic activity.

Organism	ERIA– 3	ERIA–7	ERIA–22	ERIA –27	ERIA 31	ERIA –33	ERIA–40	Culture control
Pseudomonas aerougenosa	2.2	5.0	4.1	2.3	3.0	2.4	2.2	5.0
Enterococcus feacalis	3.0	4.0	4.0	3.0	3.2	3.0	2.5	4.6
Escherishia coli	3.0	4.0	3.0	2.2	3.5	3.0	2.1	6.0
Candida albicans	2.0	3.4	4.3	2.0	3.0	2.2	1.3	5.l2
Bacillus subtillus	2.2	4.0	4.1	2.0	3.2	2.0	2.0	5.0
Klebsiela pneunomia	2.4	4.0	3.0	2.1	3.4	2.0	1.5	5.6
Staphylococcus aureus	3.0	4.0	3.0	2.1	3.4	2.0	1.5	5.2
Buffer Control	2.0	3.0	4.0	2.0	3.3	2.0	2.0	0.0

Table 7.
MTT assay to determine the cytotoxicity of protease obtained from isolates ERIA–31(enzyme I) and ERIA–33(enzyme II) on A549 cell line.

Concentration of Enzyme		O.D at 570 nm	Cytotoxicity (%)	Mean ± S.D
Protease from ERIA–31	30.0 μg	0.101	57.04	0.101 0 ± 0.001 0
	15.0 μg	0.122	48.45	0.122 0 ± 0.001 0
	7.5 μg	0.144	38.87	0.144 0 ± 0.000 5
Protease from ERIA–33	20.0 μg	0.106	55.07	0.106 0 ± 0.001 0
	10.0 μg	0.132	44.22	0.132 0 ± 0.001 0
	5.0 μg	0.151	36.19	0.151 0 ± 0.001 0
Control	–	0.237	–	–

showed maximum increase in the activity. ERIA–31 and ERIA–33 showed a 40% increase in enzyme activity when they were incubated in the presence of dithiothreitol, confirming it to be a cysteine protease. The cysteine protease active site specific inhibitor iodoacetamide at 10 mM reduced the activity of ERIA–31 and ERIA–33 50%–70% (Figure 2).

Protease was found to be most active at pH 4 to 6, 8 and 9. The enzyme showed maximum activity of pH 4 and retained nearly 80% activity at pH 9 and 60% activity at pH 5, 6, and 8 (Figure 3). Protease stability was tested at a broad range of temperatures from 4 ℃ to 50 ℃. It showed 100% activity at 4 ℃ and 8 ℃; it showed 70% activity at 10 ℃ and 28 ℃, but lost its activity completely at 37 ℃ (Figure 4). ERIA–31 and ERIA–33 showed significant bacteriolytic activity. The cell lysis was tested for 0–24 h in Elisa reader. The OD values of ERIA–31 and ERIA–33 were similar to the buffer control (Table 6). A549 cells are

Human lung adenocarcinoma epithelial cell line. Protease obtained from the two isolates was used against A549 cell lines in a 96 well plate. Protease exhibited cytotoxic effect on A549 cell line. Protease from isolate ERIA-31 showed maximum cytotoxicity against cancer cell line at 30 μg/mL concentration. Protease isolated from ERIA-33 showed cytotoxicity at 55.07 μg/mL concentrations (Table 7). Protease affected the growth of cancer cell line (Figure 5). The activity of the enzyme was concentration-dependent.

4. Discussion

In India, Western Ghats, north-eastern and northern Himalayas are rich sources of biodiversity. The studies related to the actinomycetes from these regions are very less. Hence in the present study soil samples were collected from various altitudes and different temperature zones of northern Himalayas and forty six isolates of actinomycetes were identified. Actinomycetes are good candidates for biotechnological applications[25].

By using a wide range of isolation media, culture characteristics combining physiological and biochemical characteristics, the isolates were identified. All the isolates produced filamentous type of hyphae. Some cultures produced aerial mycelium; some others produced substrate mycelium.

The colors of the mycelia were grayish and spore chains were light yellow or light grey in colour. The physiological and biochemical analyses including determination of thirty nine characteristics proved that two of the strains were similar; all the other strains differed from each other. Seong[26] reported that thirty five rare actinomycetes strains were chosen using selective isolation approaches.

The isolates belonged to one of the following genera, Micromonospora, Microbispora, Actinoplanes and Streptosporangium. In the present study only seven isolates were studied for utilization of carbon sources. Maximum number of cultures utilized dextrose and ribose. Utilization of various carbon sources by ERIA-33 indicated a wide pattern of carbon source assimilation. Bijender *et al*[27] reported similar utilization of carbon source by *Streptomyces* sp. DP2 and exhibited extensive activity over a broad pH range 4-12 with maximum activity at pH 8.

The salt tolerance studies indicated that no growth was seen with the increase in the concentration of NaCl. The isolate ERIA-33 was growing well in MNGA medium amended with NaCl. It can be placed in intermediate tolerance group. ERIA-33 showed good growth on medium amended with NaCl up to 4%. However, poor growth was observed at 5% to 12% and no growth was seen at 13% of NaCl. Several reports are available with respect to NaCl tolerance in Actinopolyspora and Saccharopolyspora[18].

In the present study, maximum amount of enzyme (126 PU/mg) was produced in the presence of 1.5% NaCl, followed by 4.5% and 3%. Salt had pronounced effect on growth and enzyme production. Mit-1 was also capable of growing in 10% salt indicating the halo-tolerant nature of the organism. The protease production was optimum (158 U/mL) with 5% w/v NaCl with a sharp decrease at 10% in *Streptomonospora alba* sp. a truly halophilic actinomycete[28].

All the isolates were screened for proteolytic activity. Only seven isolates showed good proteolytic activity. Our study showed that the metal ions were not the essential requirement for the activity, but at their addition, the enzyme showed a stimulatory effect. The isolate ERIA-33 exhibited maximum protease enzyme activity. Extracellular protease has been found in several kinds of Psychrophilic organisms, which include *E. coli*, *C. albicans*, *Xanthomonas maltophilia*[29].

The biochemical characterization of these seven isolates revealed that ERIA-31 and ERIA-33 isolates had significant protease activity. Optimum protease activity was observed at 4 ℃ in the present study. In contrast Bijender *et al*[27] reported that incubation temperature of active over a broad range of elevated temperatures 50-100 ℃ and possessed thermostability at 60-90 ℃ for up to 1 h showed significant protease activity.

In the present study pH of 8 to 9 showed significant increase in the proteolytic activity. Ghorbel-Frikha *et al*[30] pH range of 6.8 to 7.5 reported. It is evident from the present investigation that proteases from isolates ERIA-31 and ERIA-33 showed significant cytotoxic effects on A549 cancer cell line.

MTT assay is a test used to determine cytotoxicity of potential drugs. The activity was concentration dependent. The present study highlights the microbial diversity in cold environments of the northern Himalayas.

Conflict of interest statement

We declare that we have no conflict of interest.

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