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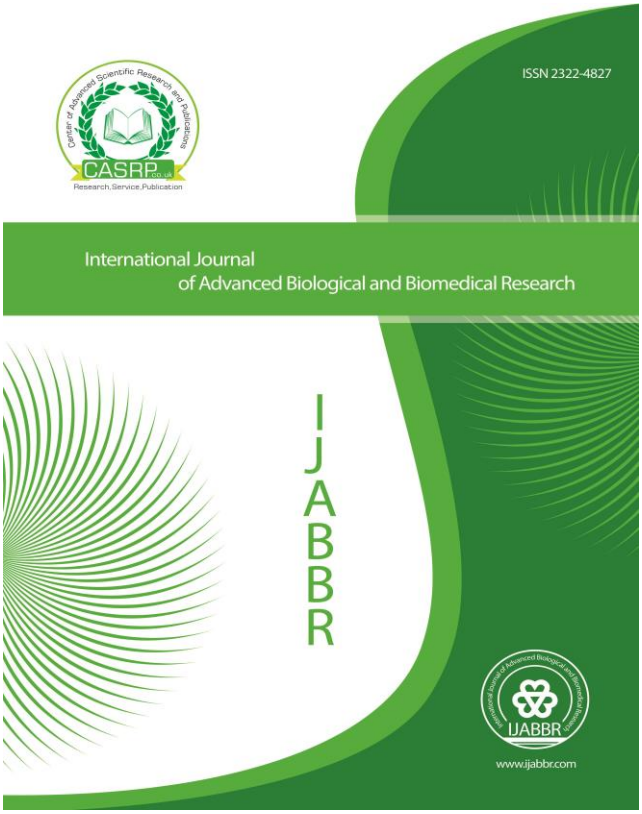

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Characterization of proteolytic enzyme secreted by *Streptomyces cinereoruber* ssp. *cinereoruber* isolated from human pleural fluid

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Abstract

Actinomycetes are an uncommon agent of human infections and its pathogenic factors are not known. The present study reports a rare case isolation of an actinomycete from a woman pleural fluid; the strain was identified by 16S rRNA gene sequence analysis. This strain was tested to produce an extracellular protease that hydrolysis gelatin, casein and hemoglobin on agar mediums. The purification of the enzyme was carried by ammonium sulfate precipitation, gel filtration and ion exchange chromatographies. The activity of protease was studied at different pH values and temperatures and in the presence of metallic ions and inhibitors. The molecular weight of the enzyme was determined by 12% Tricine SDS-polyacrylamide gel electrophoresis. The strain was identified as *Streptomyces cinereoruber* ssp. *cinereoruber*. Extracellular proteolytic enzyme was purified at 19.67 fold and a 3.0% recovery. The enzyme was characterized as having optimal activities at pH 11.0 and 50°C, it keeps more than 50% of activity at pH between 4.0 to 12.0 and it is thermostable at 30 and 40°C. Enzymatic activity is enhanced in the presence of metal ions and inhibited by EDTA and 1,10-phenanthroline. The molecular weight was 53 kDa. This study reports the first case isolation of *Streptomyces cinereoruber* ssp. *cinereoruber* from pleural fluid, the extracellular zinc-metalloprotease was proposed as candidate virulence factor.

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1. Introduction

Actinomycetes, Gram positive filamentous bacteria, are widely distributed in nature and can be found in almost all environments. Among such bacteria *Streptomyces* sp. are dominant and mainly known as important industrial microorganisms because of their capacity to produce numerous bioactive molecules, particularly antibiotics and enzymes. Previously, members of the genus *Streptomyces*, and, apart from mycetoma, were not known as a direct cause of infection, but with the increase of immunosupression, many cases of invasive infections are described (Martin et al., 1999; Riviere et al., 2012; Pellegrini et al., 2012). Proteases constitute one of the most important groups of hydrolytic enzymes. They were previously considered enzymes that only catalyze protein degradation by hydrolysis of peptide bonds. Nowadays, proteases are recognized as exceptionally important molecules that are engaged in numerous vital life processes. Because certain states of protease activity can lead to signal specific diseases (Lee and Kim, 2012). Many proteases produced by pathogenic microorganisms have been studied and the findings demonstrated or suggested that they play crucial roles in a number of pathological processes. For example, they are involved in host-tissue degradation to facilitate the colonization and infection, can influence the immune system and can even act as toxins (Armstrong, 2006; Lee and Kim, 2012). They can, also, be used as biomarkers and/or prognostic markers (Lee and Kim, 2012). This paper reports identification of *Streptomyces* strain isolated from a pleural fluid specimen of patient with persistent pleural effusion. After determination of gelatinolytic, caseinolytic and hemolytic activities the purification and characterization of extracellular proteolytic enzyme was made and proposed as pathogenic factor.

2. Materials and methods

2.1. Strain isolation and maintenance

Pleural fluid specimen was collected from a 40-year-old woman hospitalized in infectious diseases services of Constantine University Hospital Center, East of Algeria. The patient presented pneumonia with persistent pleural effusion and had a history of tuberculosis. Microscopic observation of the pleural fluid specimen revealed brunching filaments with Gram positive stain. Pure culture of actinomycetes colonies was obtained on Bennett's Agar slants. Spores were stored at -20°C in 20 % glycerol until characterization.

2.2. Molecular identification

DNA was extracted using MagNApure LC DNA isolation Kit III (PG 0212). The DNA extract was amplified with universal primers, fD1:5' AGA GTT TGA TCC TGG CTC AG 3' and rP2:5' ACG GCT ACC TTG TTA CGA CTT 3' (Eurogentec, Seraing, Belgium). The full length of 16S rRNA gene was sequenced by oligonucleotides primers: 536f: 5'CAG-CAG-CCG-CGG-TAA-TAC3', 536r: 5'GTA-TTA-CCG-CGG-CTG-CTG3', 800f: 5'ATT-AGA-TAC-CCT-GGT-AG3', 880r: 5'CTA-CCA-GGG-TAT-CTA-AT3', 1050f: 5'TGT-CGT-CAF-CTC-GTG3', 1050r: 5'CAC-GAG-CTG-ACG-ACA3'. The reaction mixture for 16S rRNA gene sequencing contains 1.5 µl BDV1 buffer, 1 µl Big Dey Terminator (version 1.1 cycle sequencing kits), 0.5 µl primers, 3 µl purified water and 4 µl DNA. The PCR program was as follows: An initial denaturation (96°C for 1 min) followed by 25 cycles of denaturation (96°C for 10 s), annealing (50°C for 5 s) and extension (60°C for 3 min). Partial fragments of 16S rRNA gene sequences were combined in a complete sequence using Sequencher program (Version 4.5). Complete sequences were compared with all bacterial 16S rRNA gene sequences available in the GenBank database, using multisequences comparison program BLAST. Multiple alignments of sequences and calculation of sequence similarity levels were carried out using Clustal W (Thompson et al., 1994). Neighbour-joining tree (Saitou and Nei, 1987) is constructed using MEGA version 4.0.2. The reliability of the tree topology was evaluated by bootstrap analysis of Felsenstein (1985) with 1000 resembling.

2.3. Nucleotide sequence accession number

16S rRNA gene sequence derived from this study is submitted to the GenBank database under accession number KF991645.

2.4. Determination of proteolytic activities

Gelatinolytic activity was tested on medium containing: gelatin, 10.0; peptone, 5.0; yeast extract, 5.0; NaCl, 5.0; agar, 15.0; pH 7.0. Caseinolytic activity was determined using skim milk agar plates containing: 5 g of non-fat dry milk and 0.5 g agar in 50 ml distilled water. Hemolytic activity was observed on TSA supplemented with 5 % (v/v) of sheep blood. The strain was punctually inoculated on the mediums and incubated at 30°C for 7 days. The medium with gelatin was flooded with mercuric chloride solution ($HgCl_3$, 15.0 g; HCl, 20 ml; H_2O, 80 ml). Zones of clearance around the colonies indicated proteolytic activity.

2.5. Protease production

A 1 ml spore suspension (10^4 to 10^6 spores / mL) was added into a 250 mL Erlenmeyer flask containing 50 mL of gelatin broth, and the flasks were incubated at 30°C and 150 rpm for 96 h. The culture medium was centrifuged at 5000 rpm to remove mycelia, the supernatant was filtered trout 0.45 µm membranes and used as a crude enzyme preparation.

2.6. Protease assay

Protease activity was determined according to Tsuchida et al. (1986) using casein as substrate. The reaction mixture, contained 250 µl enzyme solution and 250 µl of 2 % casein in phosphate buffer pH 7.0, was incubated at 40°C for 30 min. The reaction was terminated by adding an equal volume of 10 % trichloroacetic acid and the reaction mixture was then allowed to stand in ice for 15 min to precipitate the insoluble proteins. The supernatant was separated by centrifugation at 5000 rpm for 10 min at 4°C and the acid soluble product in the supernatant was neutralized with 0.4 M Na_2CO_3 solution. The color developed after adding of 10 % Folin Ciocalteau reagent was measured at 750 nm. The enzyme activity was calculated from the standard curve of L-tyrosine. Protease unit was defined as the amount of enzyme that released 1µg of tyrosine per ml per min under essay condition.

2.7. Protease purification

Ammonium sulfate was added to the crude enzyme preparation to 80 % final saturation and the mixture was incubated overnight at 4°C. The precipitated protein was obtained by centrifugation for 30 min at 10000 rpm at 4°C, was re-suspended in a 6 ml of 0.2 M phosphate buffer pH 7.0 and then subjected to a process of dialysis against the same buffer. The concentrated enzyme fraction, with ammonium sulfate saturation, was purified applying a Sephadex G-75 column (60 x 1cm). The column was first equilibrated with 0.1 M phosphate buffer (pH 7.0) then the concentrated enzyme sample was loaded and eluted with the same buffer at a flow rate of 30 ml/h. The collected 2 ml fractions were analyzed for protease activity and the positively active ones were pooled. The gel filtrated enzyme sample was subjected to an ion-exchange chromatography applying diethylaminoethyl (DEAE) Sephadex column (10 x 1cm) that was equilibrated with 0.1 M phosphate buffer (pH 8.0). The column was washed with the same buffer and eluted with a linear gradient of 0 to 1.5 M NaCl in 50 mM sodium phosphate buffer (pH 8.0). The fractions with protease activity were collected and pooled.

2.8. Protein quantification

The amount of protein was determined by the method of Lowry (1951) using bovine serum albumin (BSA) (0-0.1 mg/ml) as the standard. In addition, during the enzyme purification by column chromatographies, the protein elution profile was monitored spectrometrically at 280 nm.

2.9. Protease characterization

The activity of the protease was determined at different pH values in buffers: citrate-phosphate 0.2 M (pH 4.0–6.0), sodium phosphate 0.2 M (pH 7.0-8.0), glycin-NaOH buffer (pH 9.0-10.0) and phosphate-NaOH (pH 11.0-12.0). The pH stability of the protease was determined by incubating the enzyme in the above mentioned buffers for 1 h at 40°C. Likewise, the activity of the enzyme was determined by incubating the reaction mixture at different temperatures ranging from 30 to 80°C and the enzyme activity was measured. To determine the thermal stability, the enzyme was incubated at different temperatures (30 to 80°C) for 1 h and then relative activities were calculated. The effect of different metal ions ($CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_2$, KCl, NaCl, $MgCl_2$, $MnCl_2$, and $ZnCl_2$) and inhibitors, EDTA and 1,10-phenanthroline, on enzyme activity was checked by incubating the enzyme with 1 and 10 mM (final concentration) for 1 hour at 50°C. The relative activities were estimated with reference to control without any additive. Molecular weight of the purified protease was estimated by the method of Gagaoua et al. (2014) with Tricine SDS-polyacrylamide gel electrophoresis, using 12 % separating and 4 % stacking gels. The

samples were mixed (v/v) with the sample buffer containing 100 mM Tris–HCl (pH 6.8), 1 % (w/v) SDS, 0.75 % (w/v) DTT, 0.02 % (w/v) Coomassie Brilliant Blue R250 and 20 % (w/v) glycerol and heated for 10 min in a bath water at 75 °C. A mixture of protein molecular weight contained: ß-Lactoglobuline (18.4 kDa), Actine (42 kDa), Glutamic deshydrogenase (53 kDa), Transferrine (76 kDa) and β-Galactosidase B (116 kDa) are using as markers. After separation, the gel was stained with 4.9 mM Coomassie Brilliant Blue R-250 in 50 % (v/v) ethanol, and 7.5 % (v/v) acetic acid. Protein patterns were then visualized after destaining the gel until a clear background was achieved.

3. Results

3.1. Isolation and identification

An actinomycetes strain P.B.373 was isolated from a pleural fluid specimen with gray dry and powdery colonies. Microscopic observation revealed Gram positive, non fragmented mycelium and long (up to 50) straight spore chains. Comparative analysis of an almost complete 16S rRNA gene nucleotide sequence (1459 pb) obtained from the isolate, with the corresponding sequences obtained from the Gen Bank/ EMBL/ DDBJ databases, assigned the strain P.B.373 to the genus *Streptomyces* and found the most closely related species with a high degree of relatedness (up to 99 % of sequences similarity). The 16S rRNA gene sequence of the strain showed levels of similarity of 99.85 % to that of *Streptomyces cinereoruber* ssp. *cinereoruber* JCM 4205^T and 99.45 % to that of *Streptomyces violaceorectus* NBRC 13102^T. The phylogenetic tree shows the close association of strain P.B.373 with *Streptomyces* species and formed a monophyletic clade with *S. cinereoruber* ssp. *cinereoruber* with 87 % bootstrap support (Fig. 1).

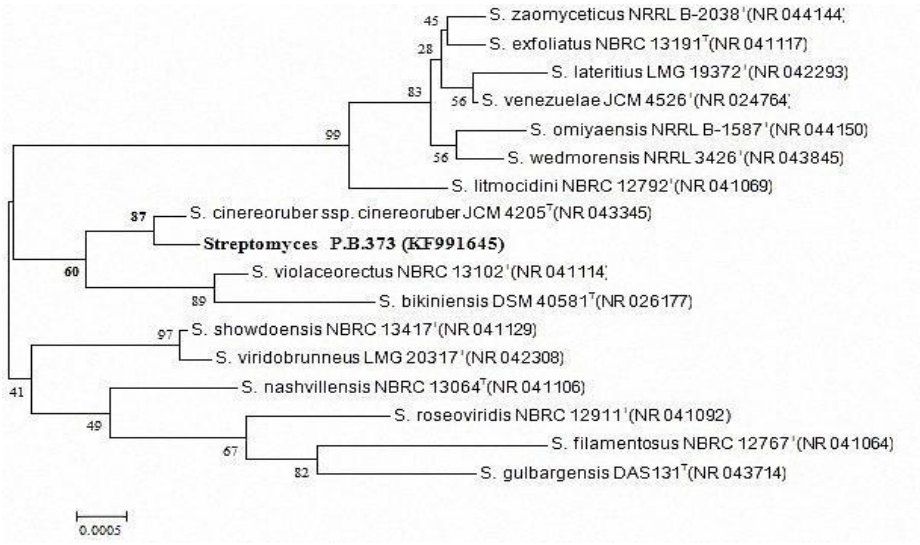

Fig. 1. Phylogenetic tree constructed by the neighbor-joining method, based on almost complete 16S rRNA gene. Sequence, showing the position of strain *Streptomyces* P.B.373 and the nearest related taxa. Numbers at nodes indicate percentages of 1000 bootstrap resamplings. Bar 0.0005 substitutions per nucleotide position.

3.2. Purification of proteolytic enzyme

Purification of proteolytic enzyme was carried out in three successive steps as shown in Table 1. The crude extract contained 326.9 mg protein; maximum activity was obtained by precipitation of 80 % saturation of ammonium sulfate. The major fraction with proteolytic activity was applied to the Sephadex G-75 column, which generated one single peak showing proteolytic activity in the eluate (Fig. 2a). The major active fraction was pooled, concentrated, and further purified using gel filtration via a DEAE Sephadex column, and one peak with specific activity was acquired (Fig. 2b). Overall, 19.67 fold purification and recovery of 3 % activity (yield) were obtained after completion of the purification steps. The specific activity of the final enzyme preparation was 788.4 U/mg protein.

Table 1
Purification steps of the proteolytic enzyme.

Purification step	Total protein (mg)[a]	Total activity (Units)[b]	Specific activity (Units/mg protein)	Recovery (%)	Fold purification
Culture supernatant	326.9	13101.45	40.07	100	1
80 % $(NH_4)_2SO_4$	44.42	2880.00	64.83	21.98	1.61
Sephadex G-75	9.39	2817.39	300.04	21.50	7.48
DEAE Sephadex	0.5	394.2	788.4	3.00	19.67

[a]Protein was measured by the method of Lowry et al. (1951), [b]Activity was measured by the method of Tsuchida et al. (1986).

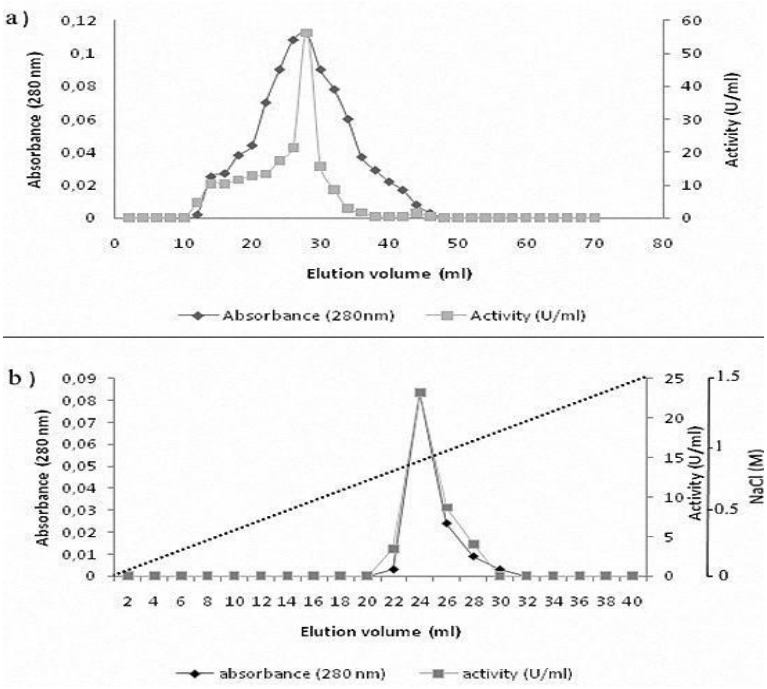

Fig. 2. Purification chromatograms of the proteolytic enzyme.
(a: Gel filtration chromatography, b: Ion-exchange chromatography)

3.3. Effect of pH

The enzyme was active in a wide range of pH. At pH 7.0, 8.0, 9.0 and 10.0, the enzyme has the best activities (89.47, 92.64, 97.77 and 97.44 %), respectively, whereas at pH 12.0 the activity was slightly decreased by nearly 10 %. Optimum activity was shown at pH 11.0. The enzyme keeps more than 50 % of activity at pH between 4 to 12 (Fig. 3).

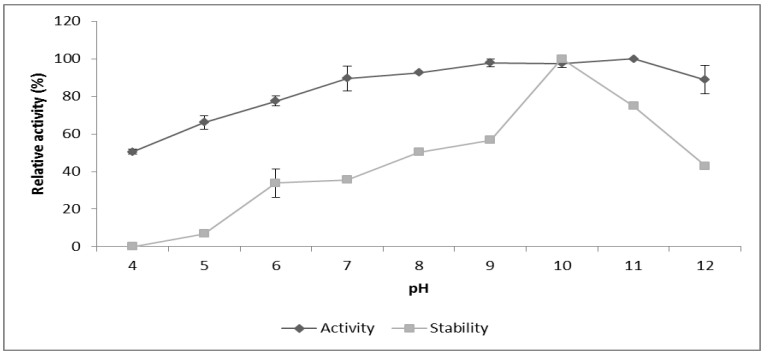

Fig. 3. Effect of pH on activity of the proteolytic enzyme.

3.4. Effect of temperature

The activity of proteolytic enzyme is more than 50 % between 40 and 60°C, optimum temperature was recorded at 50°C. The activity gradually declined at temperatures beyond 50°C (Fig. 4). The enzyme was stable at 30 and 40°C. Up to 40°C the stability decreases continuously.

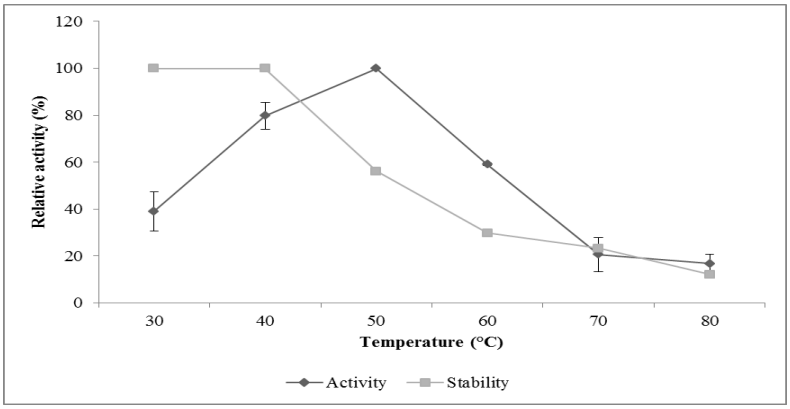

Fig. 4. Effect of temperature on activity of the proteolytic enzyme.

3.5. Effects of metals and inhibitors

The effects of various metal ions and inhibitors, at concentrations of 1 and 10 mM, on the activity of the enzyme were analyzed (Table 2). It was observed that the enzyme recovered activities by incubation with the majority of metal ions tested. These results indicate that the metal ions are located at or near the active site of the enzyme and are necessary for catalytic activity. Moreover, the enzyme was inhibited by chelators of divalent cations, EDTA and 1,10-phenanthroline, thus confirming the finding that the proteolytic enzyme of *Streptomyces* P.B.373 belonging to metalloprotease. Since 1,10-phenanthroline specially chelates Zn^{+2}, allows to suggest that the enzyme is a zinc-binding domain of metalloprotease.

Table 2. Effect of different metal ions and inhibitors on the activity of the proteolytic enzyme.

	Relative activity (%)	
Agents	**1 mM**	**10 mM**
none	100	100
$CaCl_2$	116.48 ± 6.56	107.02 ± 10.10
$CoCl_2$	98.45 ± 1.48	102.53 ± 9.58
$CuCl_2$	75.83 ± 3.76	114.07 ± 11.06
$FeCl_2$	107.29 ± 1.35	90.37 ± 3.55
KCl	110.06 ± 4.73	90.37 ± 0.48

$MgCl_2$	113.40 ± 5.83	103.96 ± 9.58
$MnCl_2$	109.17 ± 3.06	177.92 ± 5.05
NaCl	79.16 ± 1.28	78.59 ± 6.06
$ZnCl_2$	139.86 ± 1.47	105.0 ± 2.05
EDTA	85.185 0.26	56.29 ± 3.84
1,10-phenanthroline	94.14 ± 5.72	72.66 ± 0.88

EDTA: ethylene diamine tetra acetic acid

3.6. Molecular weight

Tricine SDS-polyacrylamide gel electrophoresis of the purified enzyme was performed to verify enzyme purity and determine the molecular weight. The protein migrated as a single band and the molecular mass was estimated to be 53 kDa corresponding to glutamic deshydrogenase marker protein (Fig. 5).

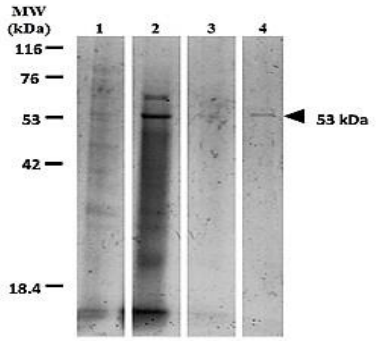

Fig. 5. Tricine SDS gel electrophoresis for molecular weight determination of the proteolytic enzyme. (Lane1: culture supernatant, lane 2: ammonium sulfate precipitation, lane 3: gel filtration chromatography, lane 4: ion-exchange chromatography)

4. Discussion

The *Streptomyces* sp. are widely distributed in natural habitats and are well known for their biologically active metabolites. In clinical practice these organisms are not considered to be a major pathogen and its virulence is thought to be low; however, it has been isolated from various clinical sources and causes many unusual infections in human and animals (McNeil and Brown, 1994). Clinical isolates of *Streptomyces* species from human are in majority cases of mycetoma and identified as *S. somaliensis* and *S. griseus* (McNeil and Brown, 1994; Dunne et al., 1998; Martin et al., 1999; Rose III et al., 2008; Pellegrini et al., 2012). However, pulmonary invasive *Streptomyces* infections have seldom been reported, isolates were identified as *S. griseus*, *S. pelletieri, S. maritimus*, *S. olivaceus* and *S. albus* (Gugnani et al., 1993; Kapadia et al., 2007). To the best of our knowledge, it is the second case that indicates the occurrence of *Streptomyces cinereoruber* from clinical specimen; the first one was from sputum samples and was considered as an unusual case of lung infection (Manteca et al., 2008).

Streptomyces sp. produces many extracellular enzymes to assure their diverse physiological demands and could provide them a mechanism to survive under different environmental conditions. For example *S. griseus* excretes a metalloprotease, SgmA, required for degradation of substrate mycelia during morphological differentiation (Kato et al., 2002). Trypsin-like protease was essential for the degradation of mycelial protein in *S. exfoliatus* (Kang et al., 1995). Moreover, proteases are essential virulence factors during all stages of the infectious process. One important function is in adherence, invasion and dissemination of the parasite, by direct host tissue destruction or fibrinolysis, proteolytic destruction of defense mediators, activation of host proteins zymogens or proteolytic activation of microbial toxins (Armstrong, 2006). During pulmonary invasion, proteases contribute as well as to delay mucociliary clearance and epithelial injury (Amitani et al., 1995). The strain *Streptomyces* P.B.373 isolated from pleural fluid specimen produces extracellular protease that hydrolyses gelatin, casein and hemoglobin. Enzyme characterization suggests that is neutral to alkaline zinc-metalloprotease.

Zinc-metalloprotease was studied as an important virulence factor in a number of diverse human pathogenic bacteria. It has been reported that *Bacillus anthracis* zinc-metalloprotease breaks down fibronectin, laminin and collagen; *Pseodomonoas aerogenosa* zinc-metalloprotease cleave fibrinogen elastin, laminin, α1-proteinase inhibitors, coagulation factors XII, IgA and IgG (Barrett et al., 2004; Chung et al., 2006); *Proteus mirabilis* produce zinc-metalloprotease that cleave IgA and IgG (Wassif et al., 1995); *Staphylococcus epidermidis* zinc-metalloproteases degrades elastin, collagen, IgG and serum α l-protease inhibitor (Teufel and Gotz, 1993); *Streptococcus pneumonia* extracellular zinc-metalloprotease removes the membrane from the epithelial glycocalyx (Govindarajan et al., 2012); *Vibrio cholera* zinc-metalloprotease degrades the extracellular matrix components fibronectin, fibrinogen and plasminogen (Vaitkevicius et al., 2008; Edwin et al., 2014). Extracellular zinc-metalloprotease is, also, characterized as toxin during *Bacteroides fragilis*, *Ralstonia picketti* and *Serratia marcescens* virulence (Marty et al., 2002; Wu et al., 2002; Chen et al., 2015), and is required for maturation of the pathogenic factor, phospholipase C (lecithinase), in *Listeria monocytogenes* (Coffey et al., 2000). Consequently, we do not exclude the possibility that proteolytic enzyme from *Streptomyces* P.B.373 is a pathogenic determinant.

In broad cases, *Streptomyces* species produces proteases at optimum 8.0 or 9.0 (Sampath et al., 1997; Chitte and Dey, 2000; Moreira et al., 2001; Ramesh et al., 2009). Whereas, optimum at pH 11.0 was also observed (El-Shanshoury et al., 1995; Hui et al., 2004). Extracellular proteases from actinomycetes generally have high optimal temperature of 40°C or above (Kang et al., 1995; Sampath et al., 1997; Chitte and Dey, 2000; Hui et al., 2004; Ramesh et al., 2009). Earlier, an extracellular alkaline protease from *Streptomyces clavuligerus*, *Streptomyces pseudogrisiolus* and *Streptomyces* sp. P3, has shown the same optimal temperature of our study (Moreira et al., 2001; Awad et al., 2013; Cheng et al., 2015), as well fibrinolytic protease from *Streptomyces* sp. (Simkhada et al., 2012; Cheng et al., 2015). Characteristics of zinc-metalloproteases from some human pathogenic bacteria are summarized in table 3. In broad cases metalloroteases virulence factors exhibit an optimum activity at neutral pH. In the present study, it is advisable to note that metalloprotease from *Streptomyces* P.B.373 provide 89.47 % of activity at pH 7.0. Stability at 30 and 40°C accord virulence factors metalloproteases from *Pseudomonas aeruginosa* and *Ralstonia pickettii*.

The molecular mass of the reported *Streptomyces* metalloprotease enzymes is in the range of 20 - 66 kDa (Mitra and Chakrabartty, 2005; Awad et al., 2013), which enclosed our result. Furthermore, the molecular mass of the purified enzyme from *Streptomyces* P.B.373 was closer as that for the metalloprotease virulence factor for *Proteus mirabilis* (55 kDa), *Ralstonia pickettii* (55 kDa), *Serratia marcescens* (56 kDa) and *Streptococcus pneumoniae* (50 kDa). This study is the first case isolation of *Streptomyces cinereoruber* ssp. *cinereoruber* from pleural fluid. Production of zinc-metalloprotease is proposed as candidate virulence factor.

Table 3
Characteristics of virulence factor metalloprotease from some human pathogenic bacteria.

Pathogenic bacteria	Characteristics*			Role in pathogenicity	References
	Molecular wight (kDa)	Optimum pH and/ or stability	Optimal temperature and/or stability		
Bacillus anthracis	18, 36, 46	7.0 to 8.0[a]	37[a], 50[b]	Cleave tissue components	Chung et al. (2006)
Bacteroides fragilis	20	ND	ND	Cytotoxic effect	Wu et al. (2002)
Listeria monocytogenes	35	7.0[a]	80[a]	Invasiveness	Coffey et al. (2000)
Proteus mirabilis	55	ND	ND	Destruction of defense mediators	Wassif et al. (1995)
Pseudomonas aeruginosa	44.6	6.0-7.0[a], 7.0[b] 7.0-8.0[a]	37-40[a], 40[b]	Colonization and invasiveness	Barrett et al. (2004)
	33		ND	Destruction of defense mediators	
Ralstonia	55	ND	37[b]	Cytotoxic effect	Chen et al.

pickettii					(2015)
Serratia marcescens	56	ND	ND	Cytotoxic effect	Marty et al. (2002)
Staphylococcus aureus	38	7.0[a]	ND	ND	Teufel and Gotz (1993)
Staphylococcus epidermidis	33	5.0-7.0[a]	37[a], 80[b]	Skin colonization	Teufel and Gotz (1993)
Streptococcus pneumoniae	50, 170, 180	ND	ND	Internalization of the bacteria	Govindarajan et al. (2012)
Vibrio cholera	35 81 and 73	ND ND	ND ND	Cytotoxic effect Cleave tissue components	Edwin et al. (2014) Vaitkevicius et al. (2008)

*all enzymes require zinc for activity, a: optimum, b: stability, ND: not determined.

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