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Characterization of a novel extracellular thermostable metalloprotease from *Pseudomonas fluorescens* migula

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Abstract
A novel extracellular thermostable metalloprotease was isolated from *Pseudomonas fluorescens* Migula, and the enzyme was estimated to have a molecular of weight of approximately 49.5kDa, pI of 4.25. The thermostability of this purified protein showed its activity even at an elevated temperature of 121°C for 15 minutes, and the LC_{50} value of purified protein was found to be 0.03µg/ml for both pre- and post-treatment against the pupae of *Cx. quinquefasciatus*. The enzyme activity was strongly inhibited by chelating agents such as EDTA and 1-10-phenanthroline, and activated by certain detergents. The metal ions Na^{+}, Cu^{2+}, Ca^{2+}, Mg^{2+},Zn^{2+} and K^{+} enhanced the action of the enzyme, other metal ions such as Hg^{2+}, Fe^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Sn^{+}, Ba^{2+}, and Al^{2+} reduced the activity of enzyme. EDTA inhibited the enzyme activity, and Zn^{2+}, Cu^{2+} ions enhanced the enzyme activity, thus shows the nature of the enzyme as a metalloprotease. The enzyme was stable in the presence of detergents, surfactants, and organic solvents and hydrolysis the casein, fibrin, gelatine, elastin, and collagen. The V_{max} and K_m values of casein hydrolysis were 0.67µmole/min and 5.10 mM, respectively. The amino acid sequence of protease is homologous with the sequence of metalloprotease M36 and the serralysin family. Thus, the results suggest that the extracellualr enzyme represents a novel thermostable metalloprotease with fibrinolytic activity.

Keywords: *pseudomonas fluorescens, cx.quinquefasciatus,* metalloprotease, serralysin, elastase

Introduction
Pseudomonas sp. is universally distributed in water, air, and soil, and frequently isolated from clinical and environmental specimens [1]. These bacterial species produce several proteases, which are one of the essential classes of hydrolytic enzymes and they are further categorized into acidic, neutral, and alkaline proteases [2]. Microbial proteases are predominantly extracellular and can be classified into four major groups based on the essential catalytic residue at active site of enzyme. They include serine proteases, cysteine proteases, aspartate proteases, and the metalloproteases [3]. Their catalytic type recognizes proteolytic enzymes, that is, aspartic, cysteine, metallo, serine, threonine, and others are yet to be classified. The most significant number of proteolytic enzymes is classified as metalloproteases [4]. Almost all metalloproteases contain one or two zinc ions, and several enzymes contain one or two cobalt or manganese ions. The HEXXH motif forming α-helix is well conserved in many mono-zinc enzymes as the active site in which the two histidine residues coordinate with the zinc ion [5]. Elastase is a zinc-containing metalloprotease which degrades variety of biologically relevant substances, including elastin, laminin, fibrin, human collagens, and immunoglobulins. The amino acid of Elastase shares its homology with neutral proteases from the Bacillus species, especially in regions considered to contain the active and zinc-binding sites of the rmoly sin [3]. *Pseudomonas fluorescens* also secretes protease, which may differ among its biotypes. A novel natural strain of *P. fluorescens* Migula obtained from a soil sample of Pichavaram flowering tree forest, Tamil Nadu, Southern Republic of India. We isolated an extracellular metalloprotease from a strain of *P.fluorescens* Migula and its mosquitocidal activities in terms of biochemical and physiological changes exerted on the larvae and pupae of the mosquito, *Cx. Quinquefasciatus* was studied [6-9]. The present study aims at characterization of extracellular enzyme produced from the bacterial strain *P. fluorescens* Migula.

Materials and Methods
Production of extracellular metalloprotease and its assay
The *P.fluorescens* Migula bacterial culture was inoculated on skim milk agar plates and incubated at 37ºC for 24h. A clear zone around the colony exhibited proteolytic activity**.** For shake flasks experiments, 6 ml of seed culture of *P.fluorescens* Migula, was grown in Glucose Peptone Salt (GPS) medium (600 ml) in 1litre conical flask containing 1.0 % (w/v) glucose and peptone, and 0.1M potassium dihydrogen phosphate (pH 7.0) and incubated at 37°C and centrifuged 500 rpm for 96 h. Protease activity was assayed by sigma's non-specific proteinase assay technique delineate by Cupp-Enyard *et al*. [10]**,** with some modifications. Casein solution (0.75%) was prepared in 50mM Tris-HCl buffer pH 7.4 and used as a substrate. The reaction mixture was made of 5ml of the casein substrate and 1ml of the centrifuged culture supernatant. After 30m incubation at 37°C, the reaction was terminated by adding 5ml of 110mM trichloroacetic acid (TCA) solution. The tubes were allowed to stand for 30m 37°C. Then, each test solution was filtered, and 1ml of the above-filtered

solution was taken into another test tube. Each test tube was incubated for 30m at 37°C after adding 5ml of 0.5M sodium carbonate, and 1ml of Folin's reagent and absorbance was measured at 660nm using UV spectrophotometer. The concentration of tyrosine produced by cultures was measured with the help of the standard graph of tyrosine obtained in the range of 10 to 100µg/ml. One unit of protease activity was defined as the amount of the enzyme resulting in the release 1µg/ml of tyrosine per minute under the assay conditions.

Precipitation and purification of the extracellular metalloprotease enzyme

The 72h culture was centrifuged at 8,000 rpm for 30m at 4°C, and the supernatant was collected for precipitation and purification of the extracellular enzyme. Enzymes in the culture supernatant of *P. fluorescens* Migula were precipitated with ammonium sulphate. The powdered ammonium sulphate was added (20–80% saturation) to the clear supernatant obtained after centrifugation with constant stirring and incubated overnight. The precipitate was collected by centrifugation at10, 000 × *g* for 30m and dissolved in the minimal amount of 20mM Tris-HCl buffer (pH 8.0). The dissolved precipitate was dialyzed against PBS, pH 7.0 at 4°C for 24h.

The culture supernatant precipitated with ammonium sulphate was dialyzed and fractionated by gel filtration using sephacryl S300 columns (Amersham-Pharmacia, Sweden) in an FPLC system. The column was first equilibrated with 50 mM PBS containing 0.1M sodium chloride, pH 7.5 at a flow rate of 1ml per minute. The loop was filled with the sample (50 µl/200 µl) and eluted at a flow rate of 1ml/ minute using PBS (0.05M), containing sodium chloride buffer (0.1M; pH 7.5), and monitored at 280nm[11].The 1 ml fractions collected were assayed for pupicidal activity against Cx.*quinquefasciatus*by introducing five pupae to each of 1ml fractions mixed with 4 ml of tap water in test tube. The active fractions showing 100% mortality of pupae were pooled, concentrated by lyophilisation and stored at -80°*C* until further use. The purity of the pooled fractions was checked and the molecular mass of the pupicidal protein was determined by HPLC using a Dupont GF-250 column. The protein concentration was estimated by modification of the Lowry *et al* [12] method using bovine serum albumin as a standard.

Effects of temperature on activity and stability of extracellular metalloprotease enzyme

The optimum temperature of purified extracellular metalloprotease enzyme was determined at different temperatures ranging from 20°C to–130°C for 15m in 0.1M borate buffer pH 7.0 containing casein (0.5%). Then the reaction mixture was incubated according to standard assay conditions for monitoring its activity levels. The metalloprotease enzyme was autoclaved at 121°C for 15m and dialyzed for 24h in phosphate buffer (pH 7.0). The autoclaved purified metalloprotease, and crude metalloprotease enzymes were subjected to 10% SDS-PAGE. The residual metalloprotease enzyme activity was determined according to the assay procedure [13].

Thermostability of the extracellular metalloprotease enzyme

The extracellular metalloprotease enzyme was autoclaved at 121° C for 15 minutes and dialyzed for 24 h in phosphate buffer (pH 7.2). The dialyzed sample was lyophilized, and the protein concentration was estimated by the method of Lowery *et al* [12]. The autoclaved protein and crude protein were subjected to 10% SDS-PAGE. Laboratory bioassay of the autoclaved protein was performed with pupae of *Cx. Quinquefasciatus*and the bioassay with enzyme without heat treatment served as control. A graph was plotted with the concentration of the protein (µg/ml) Vs. the percentage of mortality for the pre-treatment and post-treatment [11].

Effects of pH on activity and stability of extracellular metalloprotease enzyme

The action of extracellular metalloprotease enzyme was determined using different pH values in buffers at standard conditions(citrate buffer pH 6.0, Tris-chloride buffer pH 7.0-9.0, glycine-NaOH buffer pH 10-11, and Na_2HPO_4-NaOH buffer pH 12) and the enzyme activity was measured. The effect of pH on enzyme stability was determined by pre-incubating the enzyme without substrate at different pH values (6 to 12) using different buffers at room temperature for 48h. The residual metalloprotease enzyme activity was determined under standard assay conditions [14].

Effects of different metals ions on extracellular enzyme activity

The effect of various metal ions, namely Na^+, K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, Sn^{2+}, Ba^{2+}, Al^{2+} and Co^{2+}, on extracellular metalloprotease enzyme activity was determined. 5ml of 5.0 mM metal ions were added to 5 ml of 20mM Tris-HCl buffer (pH 8.0). The purified extracellular enzyme was pre-incubated with the mentioned metal ions at 60°C for 1h. Finally, the results were compared with a control without metal ion [15].

Effects of inhibitors on extracellular metalloprotease enzyme activity

Five ml of the protease inhibitors such as phenyl methyl sulfonyl fluoride (10mM),2-mercaptoethanol(1mM), iodoacetic acid (1mM),1-10 phenanthroline (0.5mM) and ethylenediaminetetraacetic acid (1mM), were pre-incubated with the purified extracellular enzyme for 1h at room temperature and The residual metalloprotease enzyme activity was determined under standard assay conditions [16].

Effects of organic solvents on extracellular metalloprotease activity

The organic solvents, namely methanol, acetone, toluene, ethylene glycol, xylene, benzene, cyclohexane, chloroform, n-butanol, Tween 20, acetic acid, glycerol, and iso-propanol were tested for their effect on extracellular enzyme activity. One ml of the solvent was added to 3ml of the enzyme solution in a screw-cap bottle and incubated at 37°C with150rpm for 30m. The remaining proteolytic activity was

Assayed using casein as the substrate as described above, in the absence (control) and presence of organic solvents. Stability was expressed as the amount of proteolytic activity that remained with the enzyme [17].

Effects of various surfactants, oxidizing agents on extracellular metalloprotease activity

The result of surfactants (Triton X-100, CTAB, and SDS) and oxidizing agents (hydrogen peroxide and DMSO) on enzyme stability were studied by pre-incubating the enzyme for 1h at 37°C and then assaying for extracellular enzyme activity. The activity of the enzyme determined without any additive was used control. The extracellular enzyme activity of the control sample (without detergent), incubated under similar conditions, was taken as 100 % [18].

Substrate specificity of the extracellular enzyme on natural substance and its kinetics

The substrate specificity of the extracellular enzyme was studied by using different natural substrates such as gelatine, fibrin, azocasein, elastin, and collagen. A 0.5ml fraction of each substrate (1.0% w/v) was prepared in 50mM Tris-HCl buffer (pH 7.4) and incubated with the extracellular enzyme at 37°C for 30m. Protease activity was calculated using standard assay conditions. The extracellular enzyme was incubated with various concentrations of casein (1 to 32 mM) prepared in 50mM Tris-HCl buffer (pH 7.4) to determine the kinetic study. Km and Vmax were calculated by linear regression from Line weaver-Burk plots [19-20]. All the experiments were conducted independently in triplicates.

N-terminal amino acid sequence analysis

The extracellular purified enzyme was isolated using a 10% SDS –PAGE. Then it is electro-transferred onto a polyvinylidene difluoride membrane (PVDF). The proteins were stained with Coomassie blue R-250 after electrophoresis. N-terminal amino acid sequences were analyzed the Edman degradation method followed by sequencing using MALDI mass spectrometry (Shimadzu, Japan). The multiple alignment and phylogenetic analyses were done to know the sequence homology [21].

Result and Discussion

The culture was harvested in 72h at which the extracellular enzyme production level was high. The precipitated enzyme was dialyzed and fractionated by gel filtration using sephacryl S300 columns (Amersham-Pharmacia, Sweden) in an FPLC system (Fig.1). The purity of the active pooled fractions was checked by HPLC using the Dupont GF-250 column. The molecular mass of the active pupicidal protein was determined on 10% SDS PAGE, and their molecular weight was found to be approximately 49.5kDa (Fig.2).

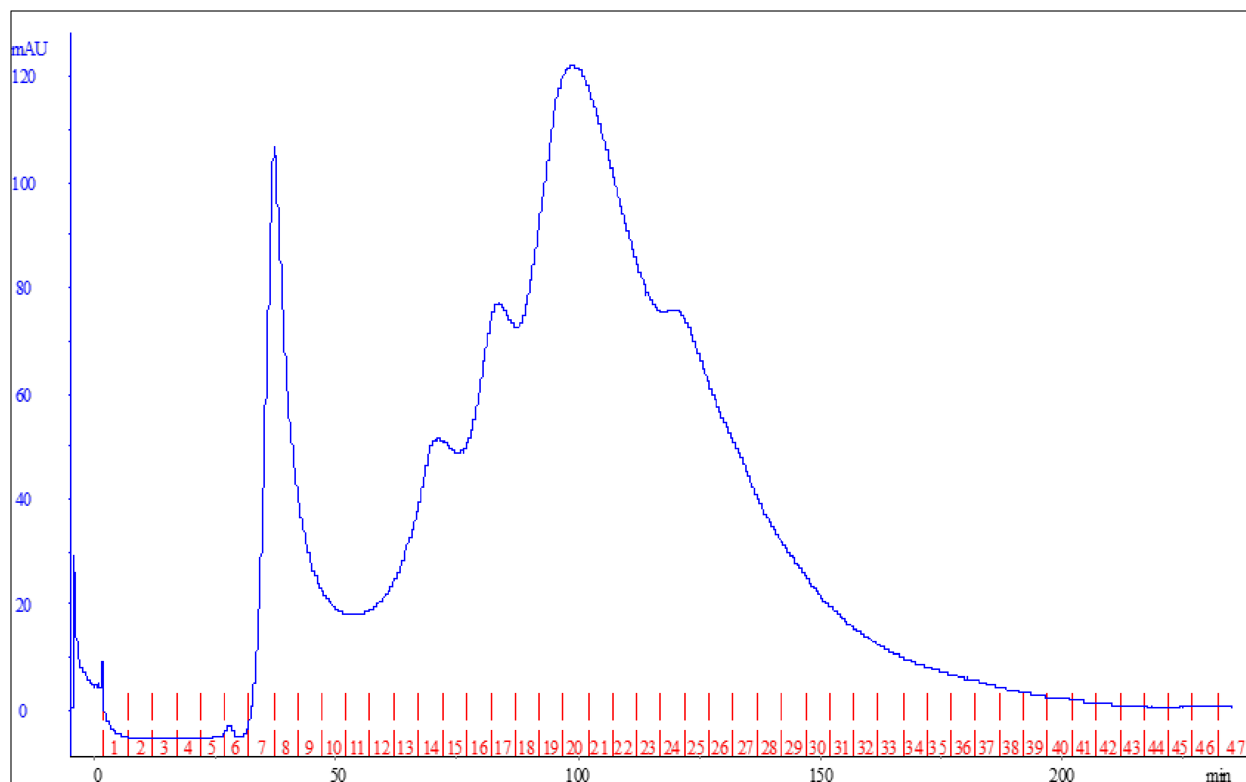

Fig 1: FPLC profile of purified fraction of extracellular enzyme of *P. fluorescens*

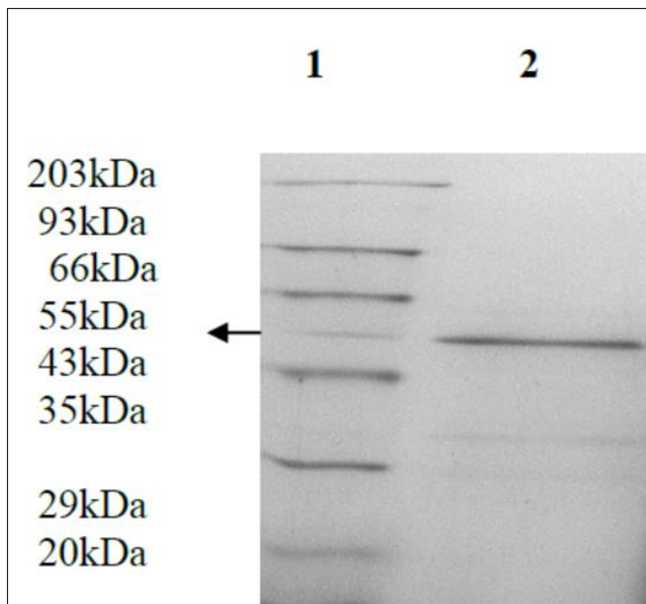

Fig 2: SDS-PAGE electrophoresis of the purified protease enzyme.

pH activity and pH stability profiles

The extracellular metalloprotease enzyme was active in a broad range of pH from 6 to 10, with an optimum pH of 7.0. The enzyme also showed 64% activity at pH 10.0 (Fig.3). The stability of protease was determined by pre-incubation of the extracellular metalloprotease enzyme at various buffers in the pH range of 4.0 to 12.0 at 37°C for 48h. Our findings have shown that the activity of the extracellular metalloprotease was stable at pH 7.0. However, the purified metalloprotease enzyme was active in a narrow pH range, and enzyme activity was decreased at pH 9.0.

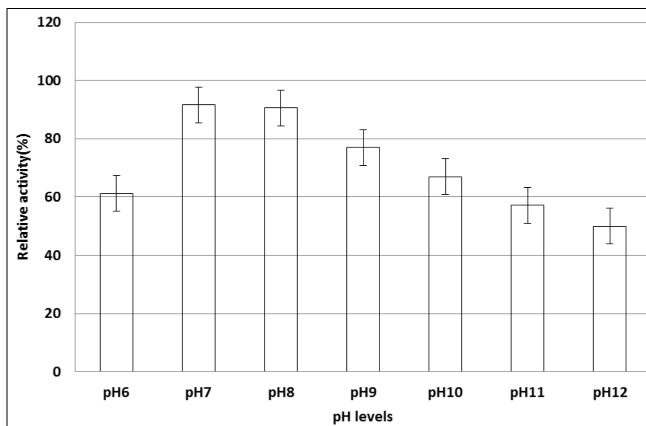

Fig 3: Effect of pH on extracellular protease activity of *P.fluorescens* Migula

Thermostability of the extracellular metalloprotease enzyme

The extracellular metalloprotease enzyme was active in a broad range of temperatures from 30°C to 121°C, with an optimum pH of 7.0. The enzyme also showed a 45% activity at 121°C (Fig.4). The extracellular metalloprotease enzyme was autoclaved at 121° C for 15 minutes and dialyzed for 24 h in phosphate buffer (pH 7.2). The dialyzed sample was lyophilized, and the protein concentration was estimated by the method of Lowery *et al.* Laboratory bioassay of the autoclaved protein was performed with pupae of *Cx. quinquefasciatus.* Bioassay with enzyme without heat treatment served as control. A graph was plotted with the concentration of the protein (µg/ml) Vs. the percentage of mortality for the pre-treatment and post-treatment.The thermostability of the FPLC fractionated pure protein was studied, and the mosquitocidal activity of pre-treatment and post-treatment purified protein was carried out against the pupae of *Cx. quinquefasciatus*. The LC50 value of pure protein was found to be 0.03ug/ml, and for both pre- and post-treatment. The LC90 value of purified protein was found to be 0.07ug/ml and for both pre- and post-treatment (Fig.5). The upper and lower confidential limits of LC 50 and LC 90 values obtained through Probit regression analysis are shown in Table 1- 2.

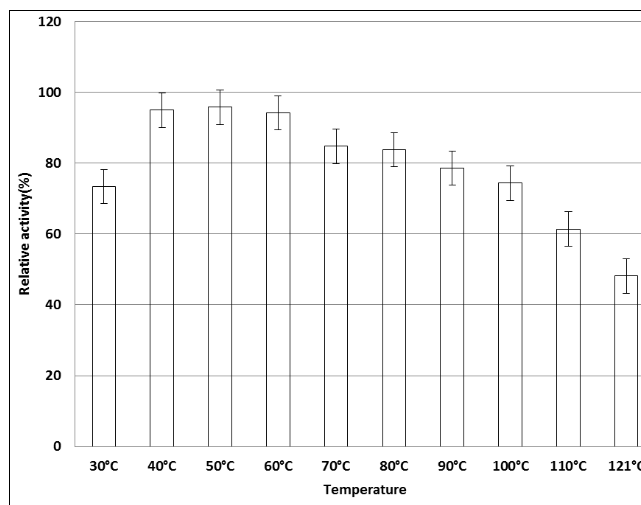

Fig 4: Effect of temperature on extracellular protease activity of *P.fluorescens* Migula

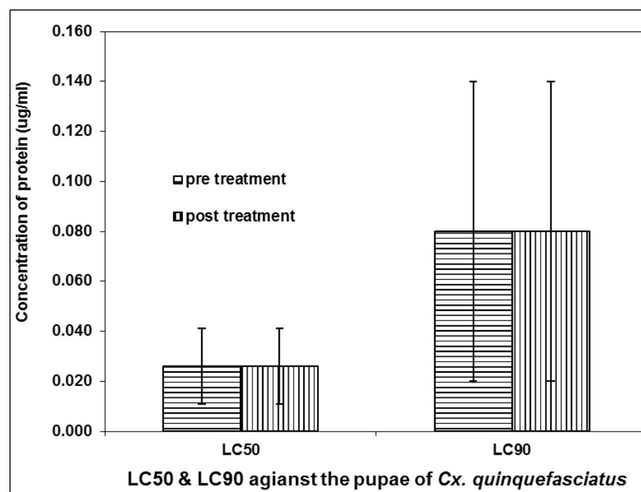

Fig 5: Stability of bioactivity of purified proteins of *P. fluorescens* after autoclaving to 121° C for 15 m

Effect of various metal ions and inhibitors

The effect of different metal ions on the purified extracellular metalloprotease enzyme was examined by pre-incubating at 40°C for 1h. A slightly enhanced activity was observed in the

presence of Na^+,K^+,Ca^{2+},Mg^{2+} and Cu^{2+} compared to the original activity, while Fe^{+2}, Mn^{+2},Co^{2+},Ni^{2+},Sn^{2+},Ba^{2+}, Hg^{2+},Al^{2+} and Zn^{+2} inhibited the activity about 15-50% (Fig.6). Among the inhibitors tested, EDTA (1mM) and 1, 10 phenanthroline (0.5mM) were able to inhibit the enzyme almost completely, and the other inhibitors, namely PMSF and β-mercaptoethanol had no considerable effects on protease activity (Fig.7). Therefore, the extracellular enzyme is suggested to be a metalloprotease.

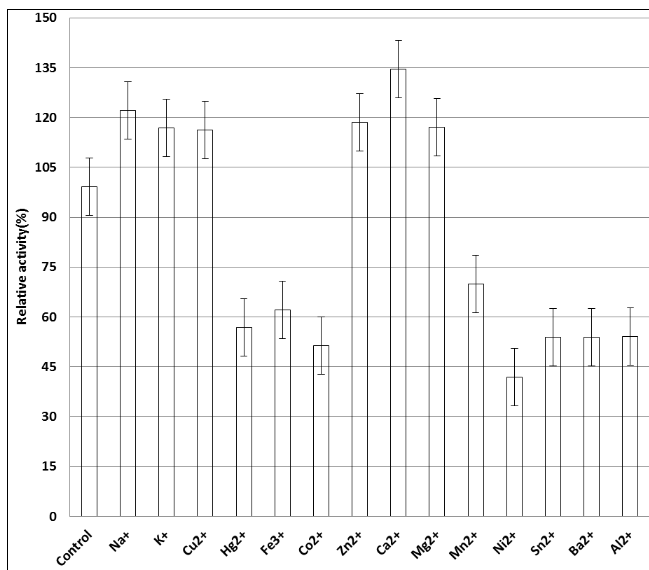

Fig 6: Effect of a metal ions on extracellular protease activity of *P. fluorescens* Migula

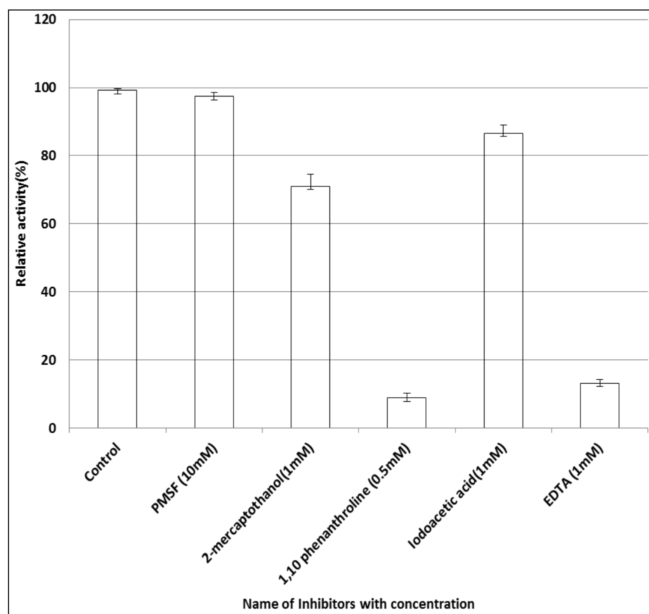

Fig 7: Effect of inhibitors on extracellular protease activity of *P. fluorescens* Migula

Effect of solvents, detergents, and other chemical agents

Two concentrations of CTAB (cetyl trimethyl ammonium bromide), SDS (sodium dodecyl sulphate), Triton X-100, and H_2O_2 were incubated with metalloprotease enzyme, and the residual activity was determined after 1h at room temperature (Fig.8). Our results showed that the effect of anionic and natural surfactants (SDS and Triton X-100) on metalloprotease activity was less than that of cationic surfactant (CTAB). Furthermore, the high concentrations of H_2O_2 (2.5%) led the metalloprotease to be 45% inactive. The metalloprotease was exposed to three different levels of DMSO. The metalloprotease was activated by increasing the concentration of DMSO (Fig.9).

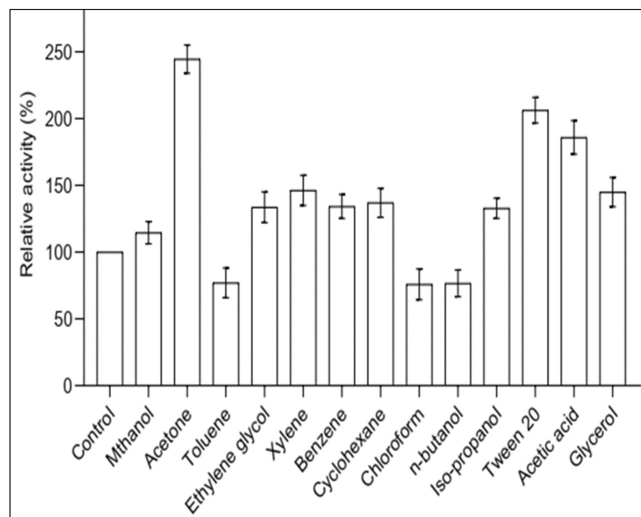

Fig 8: Effect of solvents on extracellular protease activity of *P. fluorescens* Migula

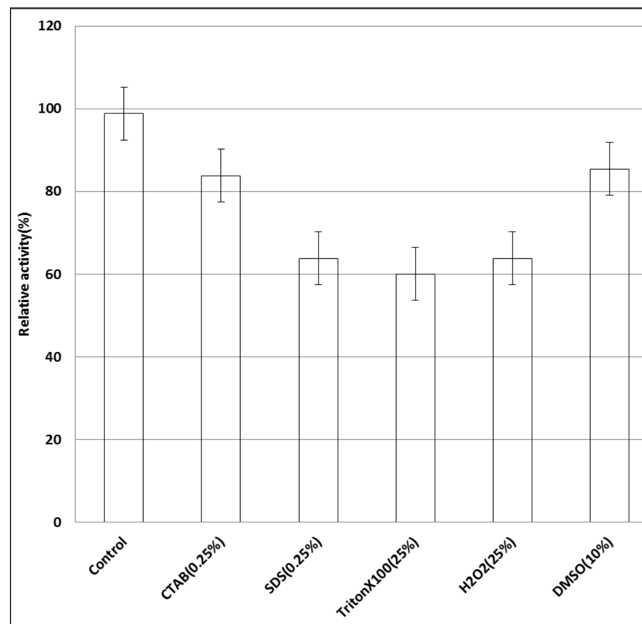

Fig 9: Effect of detergents and other chemical reagents on extracellular protease activity

Substrate specificity of extracellular metalloprotease on natural substrates

The extracellular metalloprotease enzyme produced from the *P.fluorescens* Migula strain hydrolysis various natural substrates such as azocasein, elastin, gelatine, collagen, and fibrin (Fig.10). Among the tested substrates, the purified extracellular metalloprotease enzyme showed a significant activity toward azocasein (92%). The action toward fibrin and gelatine was 88% and 85%, respectively. Since the average P-value (<0.0001) of one way (ANOVA) analysis of variance is less than P- <0.05 at 95% confidence intervals, the results of the activity of metalloprotease enzyme on natural substrates test are statistically significant. The V_{max} and K_m of Michaelis-Menten equation analysis of kinetic assay of protease activity on the different natural substrate (mM) at 95% confidence intervals are tabulated (Table 3). Therefore, the protease isolated from *P.fluorescens* Migula has a higher affinity for hydrolysis of azocasein than other natural substrates.

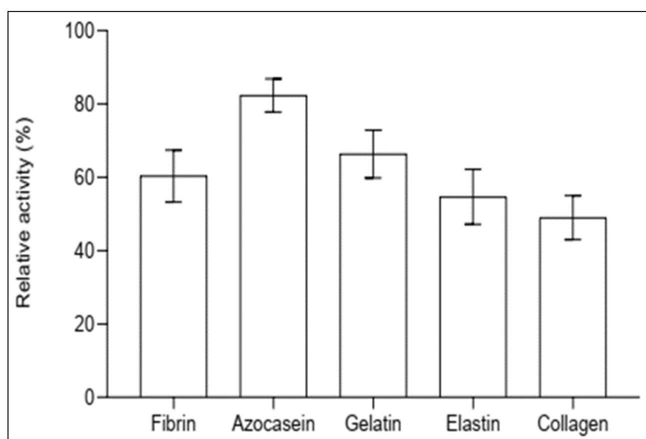

Fig 10: Effect of substrate on extracellular protease activity of *P. fluorescens* Migula

N-terminal amino acid sequence determination

The N-terminal sequence of the extracellular purified enzyme was analysed using the automated Edman degradation method following SDS-PAGE. The total sequence coverage is 18%, and the individual ions scores >53 indicate identity or extensive homology (p<0.05).Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits. The N-terminal amino acid sequence of the subunit (first 38 amino acid residues) of *P. fluorescens* Migula strain were determined to be Sdaytqvd Nflhayargg Delvng hp sytvdqaaeqilr. The sequence of 49.5 kDa extracellular protease of *P.flourescens* Migula has high similarity to those of the members of the metalloprotease family M36. Multiple alignment and phylogenetic analysis revealed the extracellular enzyme was highly identical to the residues of Zinc metalloprotease (serralysin) of *P.aeruginosa* WH6 and metalloprotease of *P. fluorescens* SBW25 respectively (Fig.11).

In this study, we investigated the characterization of a novel thermostable extracellular enzyme from *the P. fluorescens* Migula strain. The protease was purified in three steps included: ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration. The molecular weight of the extracellular enzyme was estimated to be 49.5 kDa, based on SDS-PAGE analysis. The isolated enzyme was active in a wide range of temperature activity (40-120°C). Having a high molecular weight is one of the most prominent features of the *P.fluorescens* Migula enzyme in comparison to other investigated *Pseudomonas* metalloproteases. Gupta *et al* [22] and Patil *et al* [23] reported a 35 kDa protease from *P. aeruginosa* PseA and *P.aeruginosa* MTCC 7926, respectively. Ogino *et al* [24] isolated a 38 kDa protease from *P. aeruginosa* PST-01. The enzyme exhibited high activity in a wide range of pH (i.e., from 6 to 10, particularly at pH 7.0). Neutral proteases may be active at an alkaline pH. *Pseudomonas* metalloproteases were previously reported to have a pH optimum of 8.0 for *P.aeruginosa* strains [23-24] which accommodated our results. Enzyme activity was inhibited by Fe^{2+}, Mn^{2+},Cu^{2+},Ni^{2+},Sn^{2+},Ba^{2+}, Hg^{2+}, and Al^{2+} and but was enhanced by the addition of

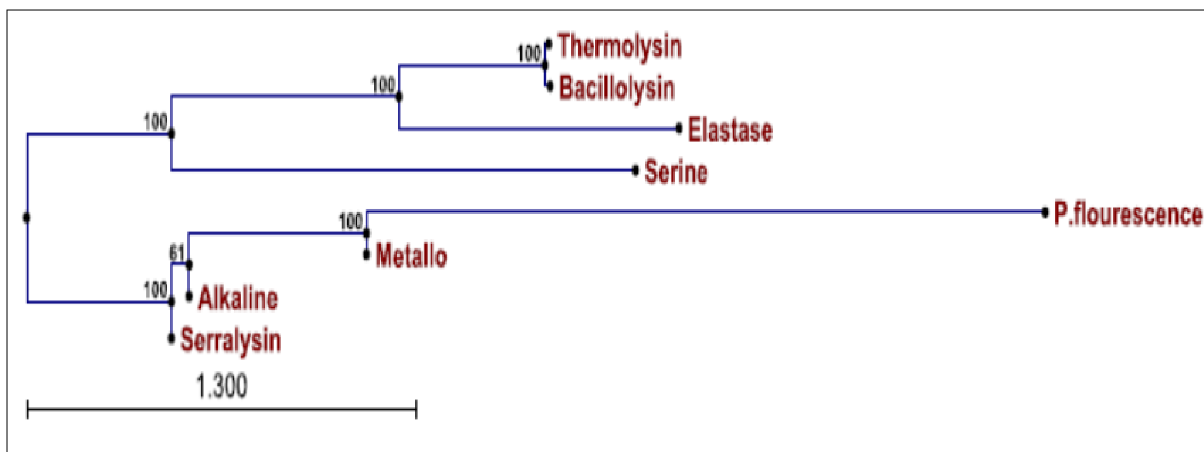

Fig 11: Phylogenetic analysisof N Terminal amino acid sequence of extracellular protease of *P. fluorescens* Migulaand closely related metalloprotease family of various microbes. Numbers at nodes are levels of bootstrap support (%) based on Neighbour joining (NJ) method. The bootstrap value below50% is not shown.

Zn^{2+},Na^{+},K^{+},Ca^{2+},Mg^{2+} and Co^{2+} ions, suggesting that the extracellular enzyme of *P. fluorescens* Migula strain is a metal

oprotease.

Enzyme proteases can be classified according to their sensitivity to various inhibitors [25]. The enzyme was strongly inhibited by typical metal chelators such as EDTA and o-phenanthroline, which indicates it as a metalloprotease. The extracellular enzyme has the similar properties of fibrinolytic enzyme obtained from *Fusarium pallidoroseum* and *P. fluorescens* strain KT1 [26-27].

Enzymes are usually inactivated or denatured in the presence of organic solvents. Our results showed that the effect of anionic and natural surfactants (SDS and TritonX-100) on metalloprotease activity was less than that of cationic surfactant (CTAB).

Previously it has been reported that non-ionic detergents such as Tween-X100 and Tween-20 improved the catalytic performance of protease, which was isolated from *Bacillus amyloliquefaciens* SP1[28].

The present protease was stable in the different solvents which were used and retained more than 70% of its activity. The protease may be stable because of the replacement of some water molecules in the enzyme with organic molecules [29].

Previously, it has been reported that the protease used in non-aqueous media can increase the solubility of substrates and products, which eventually facilitate the recovery of products [29].

The N-terminal amino acid sequence of 49.5 kDa extracellular enzyme of *P. flourescens* Migula is highly similar to the metalloprotease family M36.

The serralysin family consists of a group of bacterial metal oproteases such as alkaline protease of *Pseudomonas* strain Tac II 8 [30].

These metalloproteases are the members of the metzincin zinc family of type I secreted RTX toxins [31].

The 49.5 kDa amino acid sequence of *P. flourescens* Migula has high similarity to the sequences of serralysin family, including *S.marcescens* metalloprotease (serralysin) and *P.aeruginosa* alkaline protease.

Both metalloproteases have the extended zinc-binding motif Hexxhxxgxxh, and the third histidine of the motif and a water molecule act as the third and fourth zinc ligands, respectively. The group possessing the Xxgxxh zinc-binding motif is called the metzincins family [32].

We report significant findings of extracellular metalloprotease enzyme produced by the soil bacteria *P. fluorescens* Migula. We classified the purified protease enzyme as a member of the metalloprotease family M36 according to its N-terminal amino acid sequence and the effects of enzyme inhibitors on its activity.

It detectably hydrolyzes the M36 substrates elastin. Although it hydrolyzed fibrinogen, its N-terminal amino acid sequence was not similar to those of fibrinolytic proteases. These results, together with its catalytic properties, suggest that this extracellular protease represents a novel thermostable metalloprotease.

Further studies focusing on cloning the gene encoding this protease enzyme are required to facilitate its further characterization.

Table 1: Probit regression analysis for pure protein of *P. fluorescens* Migula - Pre- treatment

A	B	Lc50	CHISQ	UcL	LcL
3.160497	1.356337	3.881528	6.838708	4.477523	3.3646864
SE(Lc90)	SE(Lc50)	Lc90	Confidence intervals for (Lc90)		
			LcL		UcL
9.158966E-02	7.2873E-02	9.973659	8.334734		11.93486

Table 2: Probit regression analysis for pure protein of *P. fluorescens* Migula - Post- treatment

A	B	Lc50	CHISQ	UcL	LcL
3.214758	1.317297	3.877659	6.70939	4.478523	3.357411
SE(Lc90)	SE(Lc50)	Lc90	Confidence intervals for (Lc90)		
			LcL		UcL
9.375539E-02	0.730008	10.24632	8.526323		12.31329

Table 3: V_{max} and K_m value of extracellular protease enzyme activity on natural substrate

Natural Substrate	V_{max} (µmol/min)	K_m(mM)
Azocasein	0.90	7.05
Fibrin	0.75	2.68
Gelatin	0.77	2.47
Elastin	0.70	3.49
Collagen	0.67	5.10

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Conflicts of interest/Competing interests

Author declare no conflict of interest.

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