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**RESEARCH ARTICLE**

# PARTIAL PURIFICATION AND CHARACTERIZATION OF ALKALINE PROTEASE FROM *PENCILIUM OXALICUM* KRSS-S-FP10 ISOLATED FROM BEACH SOIL OF KAKINADA, ANDHRA PRADESH

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**ABSTRACT**

An alkaline protease producing strain *Pencilium oxalicum* KRSS-S-FP10 was isolated from beach soil samples collected from Kakinada, Andhra Pradesh and enzyme production was optimized under submerged conditions. Maximum enzyme production of the culture occurred at 30°C temperature and pH 9.0. glucose 28.02 U/mL and soya bean meal 23.80±0.01U/mL proved to be the best carbon and nitrogen sources respectively. The molecular weight of the enzyme determined by SDS-PAGE was found to be 28 kDa. The enzyme acted optimally at pH 9.2 and 50°C. It was thermo stable and retained full activity even at the end of 1 hour of incubation at 40°C. It was inhibited by $Cu^{++}$, $Fe^{++}$, no activity found with $Zn^{++}$ and PMSF. The enzyme retained more than 50% activity after 60 min incubation at 40°C in the presence of tannic acid, gallic acid, propyl gallate, methyl gallate and with natural plant tannins used for the tanning process indicating its suitability for application in leather processing industry.



## INTRODUCTION

Soil is the natural medium for the growth of both microbial and biological activities (Griffin, 1972). Prescott *et al*. (1993) Microbial fauna was always depends on different influencing factors such as nutrients, moisture and aeration rate pH, temperature etc. Environmental factors play vital role in the conversion of complex organic compound into reusable compounds in the nature. Plant and animal origin complex matter conversion of bioavailability of nitrates, sulfates, phosphates etc., and by different biochemical processes produce industrially useful primary and secondary metabolites such as enzymes, amino acids and vitamins and antibiotics, alcohol and organic acids (Bridge and Spooner, 2001). High depth of soil samples having much biomass (fungi) than bacteria, and also where the soil nutrients were more concentrated than surface area, which also plays crucial role (Ainsworth and Bisby, 1995). Fungi are fundamental microorganism in soil ecosystem and execute useful ecological processes to maintain the quality of human life, which facilitate economic benefits. According to the Hawks worth and Ross man, in the year (1997) and (2001) predicted that 70,000 fungi were identified and used as the industrial source out of 1.5 million fungal species on earth. Atlas and Bartha, studied in the year 1998 and describes that the count of fungi for gram soil was up to 5×103 to 9.0×106 and were accomplished to grow and survive at broad range of pH. But fungi were destructive at acid, neutral and alkaline pH. Fungi were able to uptake and utilize high concentration of substrates at favorable conditions with adequate moisture, aeration (Miyanoto *et al.,* 2002). Waksman (1922) and Warcup (1950) isolated fungi from soil by plate count method. Manivannam and Kathiresan (2007) isolated fungi from Rhizosphere soils and Charles *et al.*, (2008) isolated fungi named as *Aspergillus nidulans* from poultry farm soils. Nehra *et al.*, (2002) Kalpana Devi *et al.*, (2008) isolated proteolytic fungi from diverse sources such as garden soil and alkaline soil. Al- Falih, (2001), isolated yeast from sandy soil and soil samples from butcheries (Usama, 2008) etc.

Protease have wide applications in many industries such as textiles, detergents, food processing, meat tenderizing, animal nutrition, pharmaceuticals, paper industry and food industry and these proteases account for 60% of industrial enzymes in

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the market demand (Negi and Banerjee, 2006). According to the (1-6) the detergent industry is the major users of hydrolytic enzymes, working on alkaline pH and now more than a quarter of the global enzyme production. The huge amounts of alkaline protease used commercially and industrially (7). Alkaline proteases are used as the index of some horrible diseases such as cancer and AIDS (2). Hence clinical importance was increased and the demand for the highest alkaline protease producers was increased day by day (3). The present study aim to partial purification, studies on influencing nutritional factors on alkaline protease enzyme activity and its kinetic studies also application of alkaline protease as an alternative for leather processing.

## MATERIAL AND METHODS

### *Identification of the culture*

The fungal isolate was subjected to some gross morphological and biochemical studies *viz*., gelatin liquefaction, casein hydrolysis, tyrosine utilization and carbohydrate utilization. Gelatin was added at 15% level to sterile nutrient agar plates, inoculated with the fungal isolate and incubated at 28° for 48 h. After incubation, the plates were kept at a temperature of 2-3°C for 1h and tested for liquefaction if any. PDA, supplemented with 20% skimmed milk, was used for casein hydrolysis and incubated at 28° for 96 h for the organism.

### *Screening of Fungi by bioassay*

#### *Gelatin agar assay*

15% of gelatin dissolved in 100mL distilled water and mix with 4% of agar, sterilize the given mixture media and pour into the Petri plates and let it for solidification and inoculate fungal stains respectively and see the zone of clearance, measure the diameter according to the zone of clearance select the highest tannase activity fungal stain.

### *Production of protease under submerged fermentation*

The spore suspension was inoculated in 125 ml Erlenmeyer flasks containing 25 ml of sterilized mineral medium containing per liter (g $l^{-1}$): $KH_2PO_4$, 5; $NH_4NO_3$,10; $MgSO_4.7H_2O$,1; $CaCl_2.6H_2O$,0·1; $MnCl_2.6H_2O$,0·02; $NaMoO_4.2H_2O$, 0·01; $FeSO_4.7H_2O$, 0·125; Glucose, 2·5. Tannic acid 0.01% the pH was adjusted to 5·5 with 100 mM NaOH.

This mineral medium was autoclaved at 121 °C for 15 min. A tannic acid (Sigma) solution was adjusted to pH 5·5, filter-sterilized and added to a final concentration of 0·01 % to the fermentation medium. The cultures were grown for up six days at 320 rpm in an incubator shaker at 28ºC. The samples were withdrawn at regular intervals of one day. The biomasses were separated by the filtration through what man no-1 filter paper. The cell-free culture broth was assayed for the protease activity.

### *Effect of pH and temperature on the activity and stability of tannase*

The optimum pH was determined using the substrate in acetate buffer (pH 3.5 to 8.5). The stability of the crude enzyme was examined at different pH by incubating the enzyme with the buffers of different pH ranging from 3.5 to 9.0 for 24 h. The residual activity was estimated after incubation under the standard assay conditions and expressed as the percentage of the initial activity. For the determination of the temperature optimum, the enzyme assays were carried out at temperature ranging from 30 to 90º C. The thermal stability was investigated by incubating the enzyme at 30, 40, 50 and 60º C for 2 h. immediately afterwards the reaction mixtures were immersed in an ice bath and then the residual activities were tested under standard conditions and it is expressed as the percentage of the initial activity.

### *Enzyme assay*

Three ml of reaction mixture containing 0.5% casein in 2.95 ml of 0.1 M Tris-Hcl buffer, pH 8.5 and 0.1 ml of enzyme was incubated at 50°C. After 10 min, the reaction was stopped by adding 3ml of cold 10% TCA. After 1 hour, the culture filtrate was centrifuged at 8,000 rpm for 5 min to remove the precipitate and absorbance of the supernatant was read spectrophotometrically at 690nm. Enzyme activity was calculated by measuring mg of tyrosine equivalent released and compared with the standard. One unit (U) of enzyme activity represents the amount of enzyme required to liberate 1 µg of tyrosine under standard assay conditions.

### *Enzyme extraction*

After complete fermentation, the broth was centrifuged at 10000 rpm for 10 min at 4° to remove mycelium. The supernatant was added with ammonium sulphate to obtain 50% saturation for the precipitation of the enzyme. The precipitated proteins were pelletized by centrifugation at 8000 rpm at 4° for 20 min. The protein pellet was then suspended in 5 ml 50mM Tris-HCl buffer pH 5.5 and stored at -20$^0$C.

### *Anion-exchange chromatography*

The dialyzed tannase sample (3 ml) was filtered and applied at 1 ml/ min to a 2cc mm syringe as a column equilibrated in acetate buffer 50 mM sodium acetate pH 5.5. The column was washed with 50 mM acetate buffer pH 5.5, until the A310 was equal to that of the buffer. The column was eluted at 1 ml/min with 50 mM acetate pH 5.5 and eluted with 1 M NaCl (100mM to 1000mM collected), in a stepwise manner starting with 100 to 1000mM concentration buffer for 10 ml, followed by a linear gradient from. Each fraction was analyzed for tannase activity. Tannase-containing fractions were pooled and concentrated to 500 ml in a prior to gel-filtration chromatography. The proteins in each of the fractions were monitored by reading absorbance 310nm with UV Spectroscopy.

### *Gel filtration*

The concentrated sample (10 ml) was applied to a Sephadex G-50. And 50 ml capacity syringe used as a column for gel-filtration column pre-equilibrated with 50 mM acetate buffer with pH 5.5 and eluted with the same buffer at 1ml/ min. Fractions were analyzed for tannase activity at 310nm by using Lekha and Lonsane tannase assay method to find out the activity and protein content, followed by SDS-PAGE.

### *Molecular mass of tannase in SDS-PAGE*

SDS-PAGE was conducted using a 10% (w/v) polyacrylamide gel based on the protocol of Laemmli **(**1970). Protein bands were detected by either Coomassie blue staining. Molecular mass markers were purchased from Heline biomolecules.

### *Kinetic studies*

Enzyme was incubated with various concentrations of casein (2-20 mg/ml) in Tris-Hcl buffer (pH 9.2) at 50°C. Kinetic parameters Km and Vmax were calculated by linear regression from Lineweaver- Burk plots (Lineweaver & Burk, 1934).

## RESULTS AND DISCUSSION

### *Isolation and Screening of Protease Producing Organisms:*

A total of 74 and 69 fungi from beach soils and black soils respectively were obtained by employing different techniques of isolations Table: 1 and Fig:1. Among these, 10 from Kakinada beach soil and 9 from Vishakhapatnam beach soil and 40 fungi from Tenali black soil. Among the isolated and screened fungi 5 were selected as highest protease producers at 30$^{o}$C. A total of 74 fungi were isolated from the soil samples collected from beach soils and black soil. Single isolated colonies were selected and inoculated on casein agar plate for protease activity. The diameters of hydrolyzed zones around the colonies were calculated as a measure of caseinolytic activity of isolates. Isolates were initially screened based on zone of clearance on agar plates. Among the 74 isolates 1, 3, 7, 8 and 10 were found to have maximum protease activity and 10 was selected for further studies and pure culture was identified as *Pencilium oxalicum* KRSS_S_FP10 by RAPD method (Fig: 2).

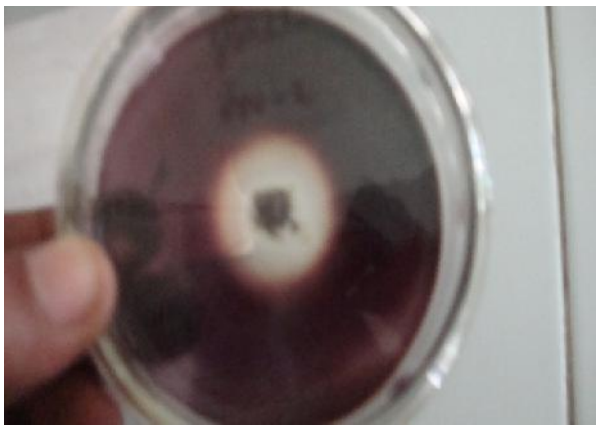

**Fig 1** screening for protease production by *Pencilium oxalicum* KRSS-S-FP10

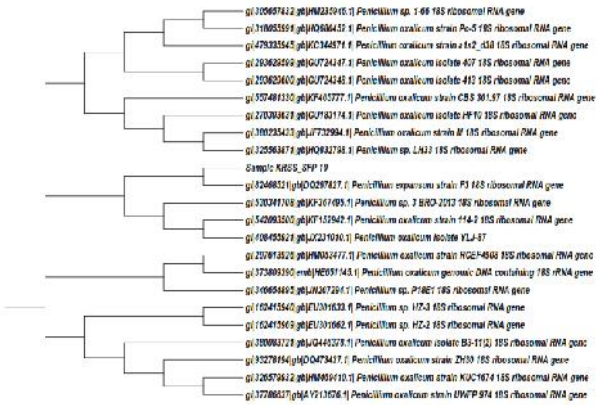

**Fig: 2** Characterization by RAPD and named as *Pencilium oxalicum* KRSS-S-FP10 isolates:

### *Purification of alkaline protease from Pencilium oxalicum KRSS_S_FP10*

Alkaline protease was purified by four step purification method includes ammonium sulphate precipitation, Dialysis and DEAE cellulose chromatography followed by sephadex G-100 gel filtration. The purification procedures of the alkaline protease secreted by the *Pencilium oxalicum* KRSS_S_FP10 was summarized in (Table: 2). The results revealed that the enzyme was purified 67.47 folds with a specific activity 420 U/mg protein after ammonium sulfate fractionation similar studies on alkaline protease enzyme fractionation at 30-70 % ammonium sulfate, there was 3.34-fold purity with 90.6% yield using *A. oryzae* (Jitender Sharma *et al.*, 2006). The enzyme was then purified with DEAE cellulose chromatography and has shown 49.45 folds protease enzyme purification with specific activity of 306.6 U/mg protein. However Ogundero and Osunlaja 13 purification studies revealed that the enzyme was recovered 26.2% by using DEAE-cellulose and Sephadex G-200 columns by *Aspergillus clavatus*. The final purification step with sephadex G-100 column chromatography showed 67.74 folds enzyme purification with a specific activity of 420 U/mg of proteins. These results indicate that the effective purification methods were applied recover the enzyme. Nevertheless, the yield of the enzyme after purification was found to be more than 60% and the remained 40% recovery of enzyme might be due to the result of autolysis of the enzyme in each purification step. Similarly purification of enzyme resulted in 49% yield and purification fold is about 1.8. And also extracellular alkaline protease produced by *B. licheniformis* AP1 was purified with 76 fold of purification and 20% yield (Tang Ming *et. al.*, 2004).

**Table 2** alkaline protease purification table

| ***Pencilium oxalicum KRSS-S-FP10*** | **Crude** | **Centrifuged** | **Precipitated/ Dialyzed** | **Ion-exchange chromatography** | **Gel fractionation sample** |
|---|---|---|---|---|---|
| Total protein | 4.603 | 3.000 | 0.463 | 0.635 | 0.481 |
| Total activity | 28.6 | 13.8 | 12.4 | 18.4 | 20.2 |
| Specific activity | 6.2 | 46 | 269.56 | 306.6 | 420 |
| Purification Folds | 0 | 7.4 | 43.47 | 49.45 | 67.74 |
| Yield of activity | 100 | 48.25 | 942.5 | 1069.9 | 1463.5 |
| Volume | 100mL | 10mL | 10mL | 10mL | 1mL |

***Determination of Molecular Weight of Purified Enzyme by Sodium dodecyl sulphate polyacrylamide gel electrophoresis: (SDS-PAGE)***

The purified protease along with standard molecular weight markers were run on SDS –PAGE. The protein in the sample migrated as a single band (Fig: 3) which indicate its homogenecity. The molecular weight of the alkaline protease was determined by comparison of the migration distances of standard marker proteins.

The molecular mass standards used were supplied by Sigma Aldrich. Depending on the relative mobility the molecular weight of the protein band was calculated to be around 22KDa. Similar studies were carried out by the Ghorbel *et al.*, (2005) and reported that the silver stained SDS-PAGE gel consists of crude, fractionated and purified fractions had shown one band corresponding to the molecular mass of 34kDa. Huang *et al.*, (2003), reported a purified dehairing protease from B. pumilus about 32kDa. Sierecka, (1997) reported similar to the relative molecular mass of 29kDa by virulent strain of *B. cereus.* Tang Ming X *et al.*, (2004) also reported more or less similar to the present research findings of the alkaline protease molecular mass when compared with the *B. licheniformis* alkaline protease molecular mass of 28kDa.

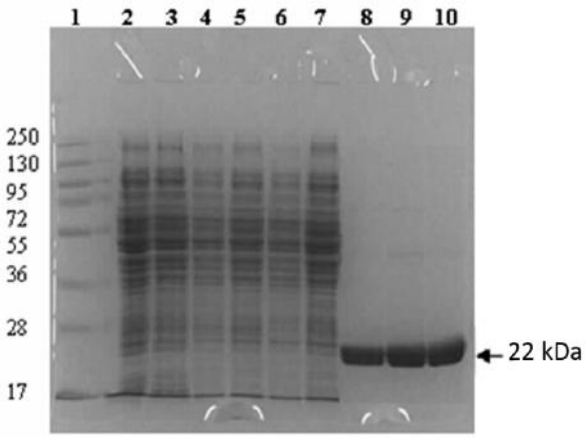


**Fig: 3** characterization of alkaline protease by SDS-PAGE

***Characterization of alkaline protease Pencilium oxalicum KRSS-S-_FP10***

***Effect of pH on alkaline protease activity***

Different pH conditions ranging from 7.0 to 11.5 were chosen to determine the influence of pH on the catalytic activity of alkaline protease enzyme. Fig:4. demonstrates the relationship, between pH and alkaline protease activity of *Pencilium oxalicum* KRSS_S_FP10 where maximal enzyme activity was obtained at pH 9.5 and the amount of consumed substrate reached 20.4±0.01 U/mg.

By increasing the pH value above 10, a gradual decrease in enzyme activity was recorded. The enzyme was stable over a broad range of pH 7 to 11.5. Ramnani *et al.*, (2005) reported that the pH optima has shown for alkaline protease in *Bacillus licheniformis*, *Serratia marcescences* (Salamone and Wodzinski, 1997), *Periserrula leucophryna* (Joo *et. al.*, 2001) and Sam sun Kim *et al.*, (2001) reported that the optimal pH of the Alkaline protease of *Bacillus cereus* KCTC 3674 was 8.0. Nilegaonkar *et al.*, (2006) has reported with *B. cereus* MCM B-326 protease were active in the pH range of 6-12.0, with optimum activity at pH- 9.0 and pH-10.6.

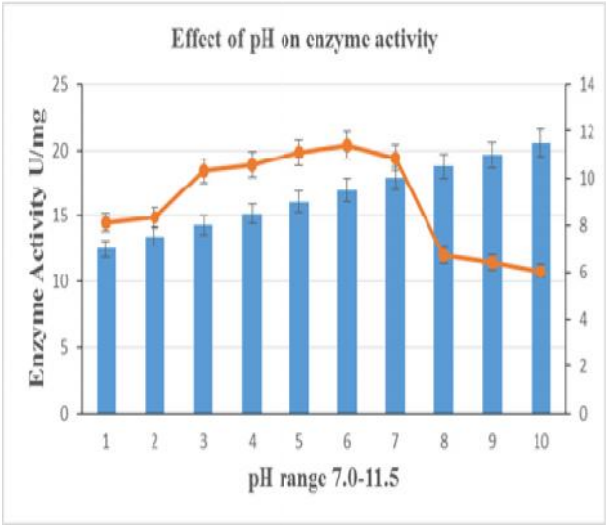


**Fig: 4** Effect of pH on alkaline protease activity

***Effect of temperature on alkaline protease activity***

An experiment was conducted to find out the degree of temperature at which optimum activity of alkaline protease was found out. A series of identical reaction mixtures were made and each was incubated at a different temperature. The range of temperatures used was from 25°C to 75°C. Fig. 4.7.2 showed a maximal enzyme activity being achieved at 50°C. The *Pencilium oxalicum* KRSS_S_FP10activity at 70°C was about 70% of that obtained at 60°C. Presumably the enzyme was denatured at 70°C, which indicates the property of its thermolability (Fig: 5). Ghorbel *et al.*, (2002) has reported similar results using *Bacillus cereus* BG1 with optimum temperature of 60°C in presence of 2mM of Ca2+ and 50°C in the absence of Ca2+. The optimum temperature of protease from virulent strain of *Bacillus cereus* was 40°C with haemoglobin as the substrate reported by Sierecka, (1997). The *B. licheniformis* RP1 has been reported that the optimum temperature in between the 65-70°C and active from 50-75°C reported by Sellami-Kamoun *et al.*, (2006).

These results indicate that elevating temperature to certain limit has a positive effect on activity; this could be attributed to increase in the kinetic energy of the substrate and enzyme molecules or/and increase the reaction rate with elevating temperatures. Beyond the optimum level of temperature, the internal energy of the molecules including translational, vibrational and rotational energy of the molecules increased, some of the weak bonds determining the three-dimensional shape of the active proteins break leading to thermal denaturation of the protein causing its inactivation. Temperatures above the optimum value also affect the protein ionization state, and the solubility of species in solution, which thus resulted in a reduction in enzyme activity (Mukherjee and Banerjee, 2006).

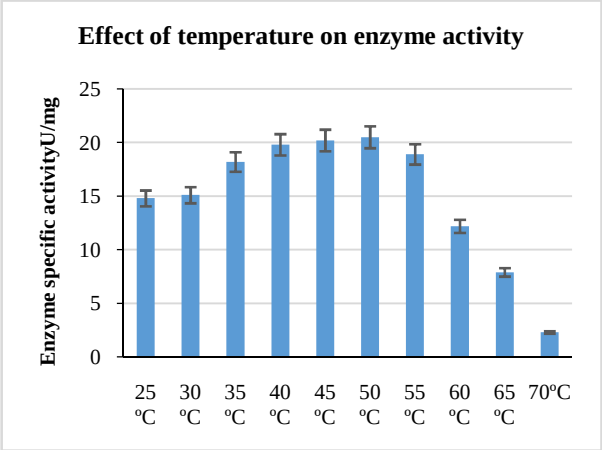


**Fig: 5** Effect of temperature on alkaline protease activity

### *Effect of metal ions on enzyme activity*

Various metal ions like $Zn^{+2}$, $Mg^{+2}$, $B^{+2}$, $Cu^{+}$, $Mn^{+2}$, $Fe^{+3}$ and $Ca^{+2}$ at 1mM concentrations were tested for their individual effect on the activity of the enzyme. Among all the metal ions studied (Table: 3), $B^{+2}$, $Mn^{+2}$, $Ca^{+2}$, $Mg^{+2}$, elevated the activity by 16.5%, 15.0%, 14%, and 12%, respectively. $Zn^{+2}$ did not show any significant effect. $Cu^{+2}$, $Fe^{+3}$, was found to be strongly inhibiting the activity. These results suggest that these metal ions apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active confirmation of enzyme at high temperature. In contrast most of the metal ions tested had exhibited a stimulatory effect on purified protease. Gupta *et al.*, (2005) has shown similar reports on protease activity was decreased to 78% with 1mM $Cu^{2+}$ but was slightly enhanced (121%) with 5mM $Cu^{2+}$ using *Bacillus sp*. Ghorbel *et al.*, (2003) has studied the effect of metal ions on Protease activity from B. cereus BG1 $Ca^{2+,}$ $Mg^{2+}$ and $Mn^{2+}$ salts increased the protease activity by 450, 285 and 157%, while $Zn^{2+}$ and $Cu^{2+}$ decreased the activity to 28% and 35.5% of the control. Beg and Gupta (2003) reported form *B. mojavesnis* slightly increased by $Mn^{2+,}$ $Cu^{2+}$ and $Co^{2+}$ up to 36% at 1mM concentration.

**Table 3** Effect of metal ions on enzyme activity

| Metal Ions | Concentration | Residual activity |
|---|---|---|
| Control | 1mM | 100 % |
| $Zn^{++}$ | 1mM | 0.2±0.01 |
| $Mg^{++}$ | 1mM | 12.8±0.02 |
| $B^{++}$ | 1mM | 16.5 ±0.01 |
| $Cu^{+}$ | 1mM | 4.2±0.02 |
| $Mn^{++}$ | 1mM | 15.0 ±0.03 |
| $Fe^{++}$ | 1mM | 2.5±0.05 |
| $Ca^{++}$ | 1mM | 14 ± 0.02 |

### *Effect of inhibitors on enzyme activity*

Sigma and Mooser (1975) studied on enzyme inhibition gave an insight into the nature of the enzyme, and its cofactor requirements and the nature of the active centre. In order to determine the nature of the protease, activities were measured in the presence of different protease inhibitors and reducing agents. Metal chelating agents such as EDTA at 25mM concentration inactivated the proteolytic activity of purified enzyme to a greater extent (75%) confirming it to be a metalloprotease. Inhibitors of serine protease (PMSF) decreased the enzyme activity to 100 (Table: 4). Similar results reported as EDTA and sodium azide decreased the enzyme activity to 100% and 20% DMSO shown effect on the protease activity reduced up to 50%. Gupta *et al*., (2005) and Ghorbel *et al.*, (2003) has revealed the results of effect of metal-complexing agents such as EDTA at 5mM concentration inactivated the proteolytic activity to 100% inhibition with Pseudomonas aeruginosa Pse A, *Bacillus cereus* BG1. Inhibition by metal chelater EDTA is a common property of all metalloproteases.

**Table 4** Effect of inhibitors on enzyme activity

| Inhibitors | Concentration | Protease activity U/mg |
|---|---|---|
| EDTA | 25mM | 5.9 ±0.01 |
| DMSO | 20% | 8.8 ±0.25 |
| PMSF | 1mM | No activity |
| PMSF | 2mM | No activity |

### *Determination of Km of alkaline protease*

Determination of the apparent $K_m$ (Michaelis constant) value of purified and crude alkaline protease was achieved through a study relating substrate concentration to the velocity of the reaction. Different concentrations of substrates were incubated with the same amounts of enzyme protein in tris buffer (pH 9.2) at 50°C for 20 minutes where the effect of substrate concentration on alkaline protease activity of *Pencilium oxalicum* KRSS-S-FP10was represented in the Line weaver Burk plot (Line weaver and Burk, 1934) of the reciprocal of initial velocities and concentrations. From this plot the apparent $K_m$ values of crude and pure alkaline protease enzyme were calculated and found to be 2.2 $K_m$ and 1.5 mM with casein. It is also clear from the results (Table: 5) It could be suggested for such finding that as a product of the reaction catalyzed by may have an inhibitory effect on enzyme activity.

**Table 5** Determination of $K_m$ of alkaline protease

| Kinetic values | Substrate casein |
|---|---|
| Crude $K_m$ | 2.2mM |
| Crude $V_{max}$ | 6200mM |
| Pure $K_m$ | 1.5mM |
| Pure $V_{max}$ | 5800 mM |

## CONCLUSIONS

The fungal isolate has shown higher protease activity comparatively reports of *Pencillin* spp. and was selected for further nutritional and influencing factors identification. The organism was identified as *Pencilium*. Further, authentic identification at Helini Biomolecules Ltd., Chennai reveled that organism belongs to *Pencilium oxalicum*. This is the first report on alkaline protease production by (*Pencilium* oxalicum KRSS-S-FP10). The alkaline protease optimized conditions were used for further industrial scale production.

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