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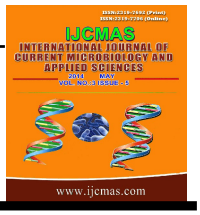

Original Research Article

Characterization of *Aspergillus oryzae* protease through submerged fermentation

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A B S T R A C T

Keywords

Protease, temperature stability, *Aspergillus oryzae*

Among the isolates *Aspergillus oryzae* KS 5 is the potential strain and study was carried out on the characterization of extracellular protease by using *Aspergillus oryzae* KS 5. The effect of pH temperature, effect of inhibitors, metals and temperature stability were studied. The highest protease activity was found to be at pH 9.0. The optimum temperature was recorded at 55°C for protease activity. The 55°C, displayed better stability and only copper induced the protease activity and it showed 121% and remaining metals and inhibitors were showed inhibition

Introduction

Proteolytic enzymes play an important part in the metabolism of almost all organisms (plants, animals, fungi, bacteria, and viruses). Investigation of proteases is a central issue in enzymology due to both their immense physiological importance and wide application in research and economical activities. Proteases are one of the most important groups of industrial enzymes and are used in a variety of industrial applications as laundry detergents, pharmaceuticals, leather products, as meat tenderizers, protein hydrolyzates, food products, and even in the waste processing industry (Joo and Chang, 2005).

This enzyme accounts for 30% of the total worldwide production of enzymes (Horikoshi, 1996).

A large number of microbes belonging to bacteria, fungi, yeast and actinomycetes are known to produce alkaline proteases of the serine type (Kumar and Takagi,1999). Proteolytic enzymes play an important part in the metabolism of almost all organisms (plants, animals, fungi, bacteria, and viruses). Investigation of proteases is a central issue in enzymology due to both their immense physiological importance and wide application in research and economical activities.

Proteases execute a large variety of functions and have important biotechnological applications. They represent one of the three largest groups of industrial enzymes and find applications in detergents, leather, food, pharmaceutical industries and bioremediation processes. (Gupta et al .2002).

Fungi elaborate a wide variety of enzymes than do bacteria and protease are among the most important enzymes produced by fungi. Fungi produce a variety of proteolytic enzymes; however, most of these are usually acidic in nature. (Fernandez, et al., 1998; Wu and Hang., 2000). Filamentous fungi are used in many industrial processes for the production of enzymes and metabolites (Adrio et al., 2003).proteases represent an important group of enzymes produced industrially and account for 60% of the worldwide sales value of the total industrial enzymes (Godfrey 1996).

The aim of the present work to characterization of the extracellular enzyme protease from *Aspergillus oryzae* through submerged fermentation.

Materials and Methods

Isolation of Fungal strains

The *Aspergillus oryzae* strains were isolated from different soils samples. Totally thirty strains of *Aspergillus oryzae*. The soils are taken from different regions from Tirupattur, Tamilnadu. Tentatively identified in the laboratory and further the strains were identified at Agarkar research Institute (ARI), Pune, India.

Screening of protease producers by plate assay

Aspergillus oryzae strains were screened for their protease production by plate assay and among the thirty isolates, *Aspergillusoryzae* were used for further studies.The screening medium is as follows. Glucose,2; skim milk,1; KH_2PO_4,1.52; KCL,0.52; $MgSO_4.7H_2O$,0.52; $CuNO_3.3H_20$, trace; ZnSo4.7H20, trace FeSO4, trace; agar, 20.0 and pH-5.0 (g/L distilled water)

Fermentation Medium

The selected *Aspergillusoryzae*KS5 were cultured on production medium.

Extraction of protease from production medium

The samples were withdrawn periodically at 24 hrs in aseptic condition. The extract was filtered through Whatman filter No.1. The clear extract was centrifuged at 2000-3000 rpm for 15 min, supernatant were used as enzyme preparation. Thus prepared crude enzyme was used for assay of protease. The crude protease enzyme were used to precipitate by ammonium salt, dialysis and further purification were carried out by ion exchange and gel filtration. The purified enzymes were used for characterization.

Assay of Protease

The protease activity was determined by the method proposed by Keay et al.(1970). 0.5 ml of suitably diluted enzyme was added to 1.0 ml of 1% casein and 0.5 ml of glycine-NaOH buffer (25 mM, pH 10.0) whole mixture was incubated at 75^0C for 10 min. The reaction was terminated by the addition of 3 ml of 10% TCA solution. The solution was allowed to stand for 10 min in cool and was filtered. To the clear filtrate, 5 ml 0.4 M Na_2CO_3 and 0.5 ml of Folin Ciocalteau reagent (FCR) was added, mixed thoroughly and incubated at

37^0C for 30 min, in dark. The absorbance was measured at 660 nm.

International units (IU)

One protease unit was defined as the amount of enzyme that released 1 µg of tyrosine per ml per minute under the above assay conditions.

Characterization of protease

Determination of optimum pH

The optimum pH of protease was determined with 1% casein (w/v) as substrate dissolved in different buffers (acetate buffer pH 4.0, Citrate buffer, pH 5.0 and 6.0, phosphate buffer pH 7.0, Tris-HCl buffer, pH 8.0, glycine-NaOH buffer, pH 9.0, 10.0 and 11.0). The activity of the enzyme in these different buffers was calculated by using methods described earlier.

Determination of optimum temperature

After the optimum pH was known, the optimum temperature of protease activity was determined by incubating the reaction mixture at different temperatures in a range from 37-55°C in 50 mM glycine-NaOH buffer (pH 9.0) for 10 min by the above mentioned analytical method.

Stability analysis

The stability of the enzyme was analyzed at room temperature and 55°C for 30 min and 60 min. The enzyme was incubated in respective temperatures and assayed at respective time.

Effect of Inhibitors and Metal ions and Inhibitors

The effect of various enzyme inhibitors (at 2mM) such as PMSF, 1-10, Phenanthroline, p-Chloromercuryl benzoate, EDTA, detergent like SDS (1%), bleaching agent like H_2O_2 (1%) and salt solution NaCl (1M). The effect of various metal ions on the protease was assayed. The metal ions were tested at 20 mM concentration. The metal ions analyzed were $HgCl_2$, $CaCl_2$,$FeCl_3$, $AgNO_3$, $ZnSO_4$, $MgSO_4$, $MnSO_4$, and $CuSO_4$.

Results and Discussion

Fungal isolates were identified as *Aspergillus oryzae* in Agrakar Research Institute, Pune. All thirty strains of *Aspergillus oryzae* produced clear zone around colony in casein plate medium; those were selected from the soil sample. Of the thirty isolates *Aspergillus oryzae* KS 5was considered to be the best and high protease producing strain. It showed 1.15 cm of cleared zone around the colony.

Determination of optimum pH

Fig.1 showed the determination of the optimum pH, Acetate (pH 4.0), Citrate (pH 5.0 and 6.0), Phosphate (pH 7.0), Tris-HCl (pH 8.0) and Glycine-NaOH (pH 9.0, 10.0 and 11.0) buffers were used. The highest protease activity was found to be at pH 9.0. The optimum pH of protease from *Penicillium janthinellum* protease was estimated to be 6.5 and from *Neurospora crassa* protease showed 6.5 (Abiram et al., 2011). Protease from Mucor, described by Maheshwari et al., (2000) also exhibited low optimum pH. Our results are agreement with Abiram*et al*., 2011.

Determination of optimum temperature

Fig 2. Showed the activity of the protease was determined at different temperatures ranging from 37°C to 55°C.

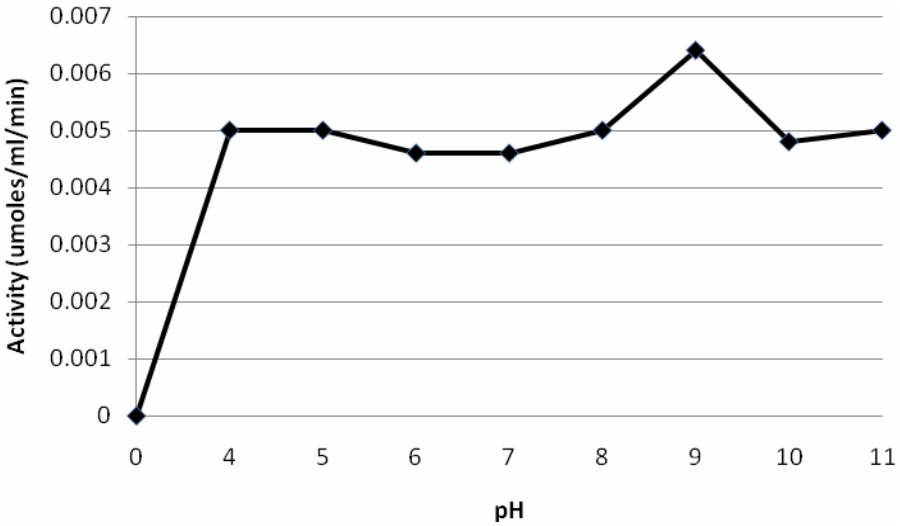

Fig.1 Determination of Optimum of pH

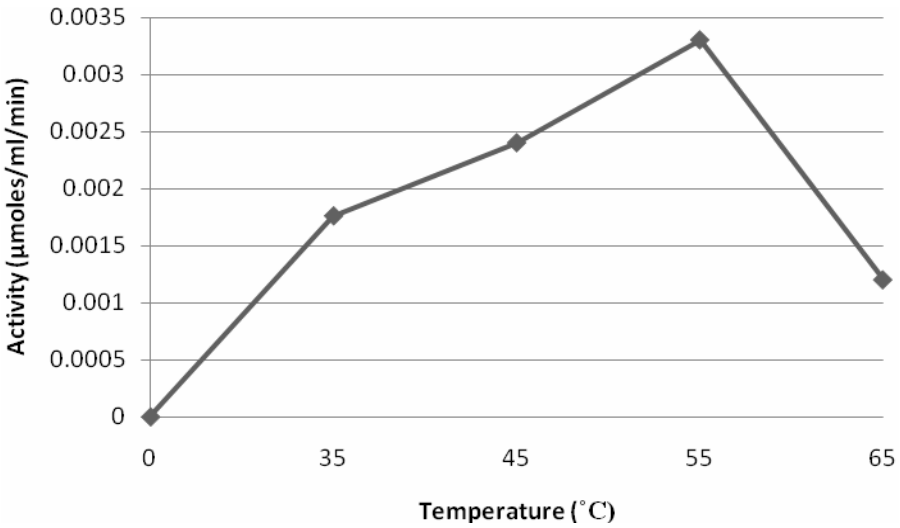

Fig.2 Determination of optimum temperature

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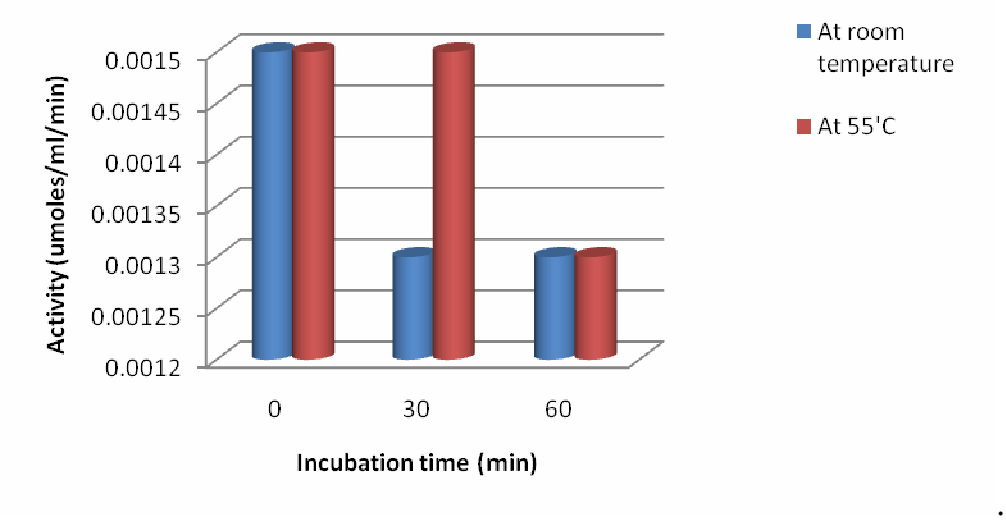

Fig.3 Analysis of temperature stability of protease

Table.1 Effect of metal ions on enzyme activity

Metal ion	Residual activity (%)
Control	100
$HgCl_2$	0
$CaCl_2$	2.74
$FeCl_3$	0
$AgNO_3$	0
$ZnSO_4$	4.57
$MgSO_4$	0
$MnSO_4$	0
$CuSO_4$	121.77

Table.2 Effect of inhibitors and other compounds on enzyme activity

Inhibitor	Residual activity (%)
Control	100
SDS	6.2
NaCl	2.4
H_2O_2	4.12
PMSF	4.46
1-10, Phenanthroline	0.34
p-Chloromercuryl benzoate	1.37
EDTA	1.37

The optimum temperature was recorded at 55°C for protease activity. The enzyme activity gradually increased up to 55°C. The proteases from the genera *Aspergillus* (coral et al., 2003, Tunga et al., 2003) and from *Penicillium* sp. (Germano et al.,2003) showed optimum activities at lower temperatures 40°C and 45°C respectively. Our results were coincides the results of Germano et al., 2003.

Analysis of stability

Fig.3 represented that thepurified protease analyzed for its stability at room temperature and at 55°C for 30 and 60 min. The results indicated that the enzyme looses its maximum activity at room temperature with in 30 min, but at 55°C, displayed better stability. But at after 60 min, it was observed that even at 55°C, enzyme looses significant activity. *Aspergillus parasiticus*, which maintained 100% of activity at only 40°C for 1 hour (Tunga *et al* .,2003) and it will agree with these results.

Effect of Inhibitors and Metal ions

Table 1 and 2 showed the inhibition studies of the enzyme purified up to nowgave a unique inhibitors and inducers list. The purified enzyme activity was induced up to 121% by Cu and all other metal ions were round to be inhibitors. PMSF did inhibit the enzyme, so the enzyme is a serine protease. Enzyme did not display activity in presence of SDS, indicating the susceptibility of the enzyme for detergents. Enzyme was not saline stable and bleach resistant, as it lost activity in presence of NaCl and H_2O_2.

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