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Production and characterization of an acid Protease from a local *Aspergillus* Sp. by Solid substrate fermentation

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ABSTRACT

The study aimed at isolating a potential acid protease producing fungal strain from local soil source. The fungal strains were isolated from garden soil on the basis of clearance zone on casein-glycerol agar flooded by coomassie blue stain. Aspergillus sp. showing maximum clearance on casein agar plates (pH 5) was selected for further studies. Optimization of various factors influencing maximum enzyme production by Aspergillus sp. using solid state fermentation was investigated. Optimum fermentation conditions for enzyme production were - substrate (wheat bran & gelatin; 1%, w/v), fermentation time (120 h), moisture content (20 %), growth pH (5.0) and temperature (30ºC). Wheat bran supplemented with nitrogen sources viz., gelatin and potassium nitrate showed 15-17 % increase in enzyme productivity. However, supplementation with additional carbon sources or salt solution had no profound influence on enzyme productivity. The crude acid protease of Aspergillus sp. showed pH and temperature optima of 5 and 50°C respectively. An increased enzyme activity was observed in presence of Ca^{2+} and Mg^{2+}. The enzyme showed broad substrate specificity. Thermostability of this enzyme at 50°C for 30 min throws light on its potential for applications in food industries.

Key words: *Aspergillus* sp., acid protease, characterization, enzymatic potential, optimization.

INTRODUCTION

Proteases with high activity and stability in acid pH range have important industrial applications, especially in the food processing industry, such as dairy industry as milk clotting agents for the manufacture of cheese [1] or to improve food flavours [2]. Acid proteases have been isolated and characterized from mammals, plants, bacteria and fungi [3]. A considerable number of *Aspergilli* species are known to produce extracellular acid proteases such as *Aspergillus niger* [4], *A. oryzae* [5], *A. awamori* [6], *A. fumigates* [7] and *A. saitoi* [8]. These enzymes are predominantly extracellular, isolated in active form from the culture medium and some of them are available in

commercial scale [9] several fungal acid proteases called aspergillopepsin have been purified and characterized from *Aspergillus* strains [10,11].

Solid-substrate fermentation (SSF) has the potential for the higher protease yield [12,13]. Economically this type of fermentation possesses many advantages, including superior volumetric productivity, use of simpler machinery, use of an inexpensive substrate, simpler downstream processing, lower energy requirements and low waste water output [14].

Growth conditions and nutrients promote high yields of microbial enzymes. However, use of pure carbon sources such as dextrin, fructose, glucose, lactose, maltose and starch are not economical [15,16], which can be replaced with economically available agricultural by-products [17] or industrial amylaceous substances as carbon substrates [1, 18-20].

The isolation and screening of microorganisms from naturally occurring acid habitats are expected to provide new strains producing enzymes active and stable in acid conditions.

Hence an attempt is made on the production of economically viable acid protease from a cheap agro residue, wheat bran, fermented by a local strain of *Aspergillus* sp. The study also includes partial characterization of the crude enzyme.

MATERIALS AND METHODS

Isolation of acid protease producing strain:
Fungal strains isolated from local garden soil samples by serial dilution method (Aneja, 1993) were screened for acid protease production using a qualitative plate medium at pH 5 in Petri plates (90 mm) diameter containing (in %): glycerol 0.5, casein 1, yeast extract 0.3, NaCl 0.5 and agar 2. The medium was inoculated with a loopful of fungal spores and incubated at room temperature for 48-72 hours. The plates were then stained with Coomassie Brilliant Blue R-250 for 2 h followed by destaining overnight to observe the hydrolysis zone by acid protease [21]. The fungal strain giving maximum clearance zone was identified as *Aspergillus* sp. on the basis of colony morphology and microscopy and was selected for further studies.

Screening of substrates
11 different substrates including 8 cereals and 3 agro industrial residues were screened for protease production by solid state fermentation (skimmed milk, soya bean meal, casein, green gram flour, red gram flour, wheat flour, wheat bran, pea-nut flour, peas flour, roasted gram flour, black gram flour).

Optimization of process parameters
Various process parameters influencing protease production such as fermentation time (24, 48, 72, 96, 120, 144 and 68 h), initial moisture content (10, 20, 30, 40 and 50 %) and fermentation temperatures (20, 25, 30 and 37 °C) were optimized. The medium was supplemented with different carbon sources (dextrose, fructose, sucrose, maltose, lactose and starch at a concentration of 1%), different organic nitrogen sources (casein, soya meal, skimmed milk, gelatin, yeast extract and albumin) and with different inorganic nitrogen sources (ammonium

sulphate, potassium nitrate, diammonium hydrogen phosphate and ammonium chloride at a concentration of 0.5 %). SSF was also performed under optimized conditions.

Solid state fermentation

A known quantity (5 g of the substrate) was taken in a 250ml Erlemmeyer flask, moistened with salt solution [composition (%,w/v): sodium nitrate 0.2, potassium dihydrogen phosphate 0.1, magnesium sulfate 0.05, potassium chloride 0.05, ferrous sulfate trace, zinc sulfate trace, pH 7.0 to achieve the desired moisture content, sterilized at 121.5^{o}C at 15 psi for 15 mins, cooled, inoculated with 1ml of fungal spore suspension (10^{8} spores/ml) and incubated at 30°C for 72 h, unless otherwise mentioned.

Enzyme extraction

After the desired incubation, a known quantity (5 g) of the fermented material was mixed with 30 ml of 0.1M phosphate buffer and homogenized by shaking for 30 min and filtered through cheese cloth. Cell free supernatant was obtained by centrifuging the extract at 10,000 x g for 30 mins and filtering through Whattmann filter paper. The volume of filtrate which contained the crude enzyme was measured and used for the protease assay.

Assay for acid protease

The acid protease activity in crude enzyme extract was assayed according to the modified method of Anson (1938) using BSA as substrate [22]. Reaction mixture containing 0.5 ml of enzyme solution and 0.5 ml of 1 % (w/v) BSA in 0.2 M Phosphate buffer was incubated at 30° C for 10 min. Except where specified, enzyme reactions were carried out at pH 5 .0. The enzyme reaction was stopped by adding 1 ml of 10 % trichloroacetic acid containing 0.22 M acetic acid and 0.33 M sodium acetate. The reaction mixture was allowed to stand for 30 min at 30 °C and then was filtered. To 2 ml of the filtrate, 5 ml of 0.55 M sodium carbonate was added, followed by the addition of 1 ml x 3 times diluted phenol reagent. The blue colour was measured at 660 nm by using a spectrophotometer. One unit of activity was defined as the amount of enzyme producing a change of absorbency equivalent to 1 µg of tyrosine per min, under the above conditions.

Characterization of the crude enzyme

Temperature optima

Temperature optima of the protease by determined by incubating the reaction mixture at different temperatures (16, 24, 27, 37, 45 and 50 °C).

pH optimum

The pH optimum of the acidic protease enzyme was determined using buffer solutions of different pH using the following buffers at 0.5 M concentration: acetate (pH 4-6), phosphate (pH 7.5, 8.5) and Tris-HCl (pH 10).

Hydrolysis of protein substrate

Protease activity with various protein substrates including BSA, casein, egg albumin and gelatin was assayed by mixing 100µl of enzyme and 200 µl of assay buffer (acetate buffer, 0.5 M, pH 5) containing protein substrates (2 mg/ml). After incubation at 50 °C for 30 mins, the reaction was stopped by adding 200 µl of 10 % TCA and allowed to stand at room temperature for 10

minutes. Undigested protein was removed by centrifugation and peptides released were assayed. Specific protease activity towards BSA was taken as control.

Effect of metal ions

Different metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+}) at a concentration of 0.01M were used to study their effect on enzyme activity.

Bovine haemoglobin Assay

2.2 % haemoglobin was adjusted with Hydrochloric acid to pH 1 and allowed to stand at room temperature for 2 h. To 1ml of denatured haemoglobin, 1ml of 0.5 M Citrate Buffer (pH 2.5) and 1ml of enzyme extract was added and incubated at 38°C for 20 mins. The reaction was stopped by addition of 3 ml of 5 % TCA and filtered. Absorbance was measured at 280 nm [22].

Chemicals used and Statistical Analysis

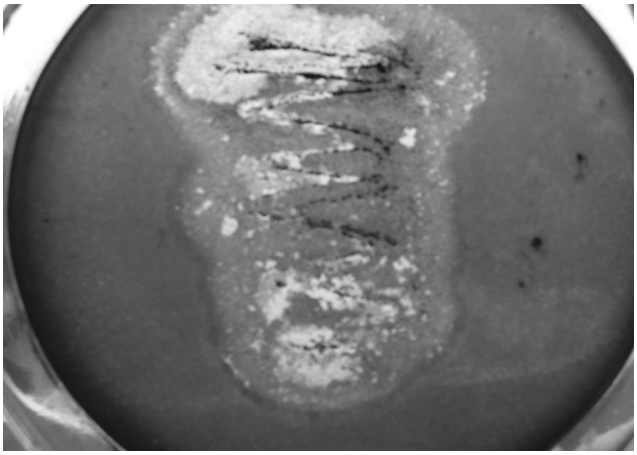
All analytical reagents and media components were purchased from Hi-Media (Mumbai, India). All the experiments were carried out independently (in triplicates) in 250-mL Erlenmeyer flasks. The data represented here are in the form of mean ± SD. All the values were subjected to one way analysis of variance (ANOVA) and significance is presented as Duncan's multiple range test results in the form of probability ($P \leq 0.05$) values, which were obtained using MSTAT software.

RESULTS AND DISCUSSION

Isolation and screening of acidic protease producers

Of the total 15 fungal strains isolated from garden soil which tested positive for acid protease, *Aspergillus* sp showed maximum hydrolysis zone on casein agar at pH 5 and hence was selected for substrate screening (Plate 1). The hydrolysis zone produced on the casein plate could be related to the amount of protease produced by the fungus [23].

Plate 1 showing acid protease production by *Aspergillus niger*.



Screening of substrate

In SSF, the solid substrate not only serves as an anchorage but also supplies nutrients to organisms. Therefore selection of a suitable solid substrate is a critical factor and thus involves screening of substrates (agro industrial materials) for microbial growth and product formation. Of the different substrates tested for growth and protease production by *Aspergillus sp*., black gram, peas, red gram, roasted gram and all other substrates as mentioned in Table 1 supported growth and enzyme production, whereas gram flour did not. Wheat flour, wheat bran and soya flour proved superior to other substrates as they supported a high titre of protease activity of 320, 280 and 160 units/g respectively (Table 1). Though wheat flour supported highest protease production by this strain, wheat bran was used for all the subsequent studies for economical reasons.

The coarser variety of bran is considered to be a better substrate as it does not form a compact mass and permits better air circulation, heat dissipation and penetration of mycelia. Also, it is a better prospect economically since it is cheaper than the finer variety of bran. Extended sterilization allows this coarse wheat bran to be used in a form such that nutrients present in it are easily available to the organism [14].

Table 1. Screening of different substrates for acid protease production by *Aspergillus* sp. using SSF

Different Substrates	Activity(U/g)
Pulse flour	
Peas	40
Black gram	0
Peanut	120
Roasted gram	40
Gram	40
Green gram	120
Agro-industrial products	
Soya	160
Wheat flour	320
Wheat bran	280
Industrial byproduct	
Casein	80

Optimization of process parameter

Incubation Time

The results for optimal incubation period indicated that protease yield increased gradually and attained high titer of enzyme activity (280 U/g) at 120 h of incubation. Further incubation reduced the yield. (Table 2). Chakraborty et al. (1995) isolated a new fungal isolate identified as *Aspergillus niger* var *tieghem* with the capacity to produce enormous amounts of a highly acidic extracellular protease on solid substrate [24]. The organism could elaborate enzyme activity when wheat bran was used as the sole substrate for production.

Initial Moisture Content

Increase in moisture level is believed to reduce the porosity of the wheat bran, thus limiting oxygen transfer, while lower moisture content causes reduction in solubility of nutrients of substrate, lower degree of swelling. Hence, an optimal level of moisture is required for maximum enzyme productivity. High enzymatic titer (240 U/g) was attained when the initial

moisture level was 22.4 % (Table 2). It is known that water content of a medium has a profound influence on the production of products by microorganisms. In a solid state fermentation the water content of the substrate is greatly influenced by the absorbing capacity and capillary forces of the substrate, the growth temperature, the amount of metabolic heat generated, the quantity of moisture evolved and growth requirement of organisms [14]. Lindenfelser and Ciegler (1975) noted for ochratoxin A production by *Aspergillus ochraceous* in solid state fermentation that the wheat bran moisture content was the most critical of all the fermentation conditions studied [25]. Wang *et. al.,* (1974) reported that 50- 63 % was sufficient for acid protease production by strains studied by them under solid state fermentation conditions [26]. 52 % moisture content of the wheat bran at 28°C and 32°C provided good environment for the production of acid protease at 54-72 h of growth by *Aspergillus niger* var. *tieghem.* However, the productivity of the enzyme was highest at relatively higher temperature (37°C) with 70 % moisture content, although it took longer (126 h) than the optimal time (54-72 h) of growth [24].

Table 2. Effect of various physical parameters on acid protease activity of *Aspergillus* sp

Physical parameters		Protease activity ($U*mL^{-1}$)
Incubation period (h)	48.0	$0±0^{e}$
	72.0	$5±0.5^{d}$
	96.0	$27±2.52^{c}$
	120.0	$281±3.61^{a}$
	144.0	$125±4.04^{b}$
Moisture (%)	10	$120±5.0^{d}$
	20	$278±2.89^{a}$
	40	$206±5.29^{b}$
	60	$149±5.13^{c}$
	80	$70±4.04^{e}$
	100	$51±4.04^{f}$
pH	2	$0±0.0^{e}$
	3	$10±0.64^{d}$
	4	$30±2.0^{c}$
	5	$279±2.52^{a}$
	7	$140±2.0^{b}$
Temperature(°C)	20	$112±2.52^{d}$
	25	$210±2.0^{c}$
	30	$281±3.21^{a}$
	37	$249±1.73^{b}$

*Values are mean ±standard error from 3 replicates in each group, Values not sharing a common superscript letter(s) differ significantly at p<0.05(DMRT), * One unit of activity was defined as the amount of enzyme producing a change of absorbency equivalent to 1 µg of tyrosine per min at pH 5.0 and 30°C.*

Fermentation pH

The optimum pH for acid protease production by *Aspergillus* sp. in wheat bran was found to be 5.0 (Table 2). pH below and above 5.0 affected the enzyme production indicating the acidic nature of the protease. Similar observation was made by Ikramul *et. al.,* (2003) in *Rhizopus oligosporus* HIS_{13} under SSF conditions [27].

Fermentation Temperature

Temperature is also one of the key factors which influences the outcome of solid state fermentation system. Our studies on growth and enzyme production at 20, 25, 30 and 37°C indicated that although luxuriant growth occurred at all of these temperature , protease

productivity (280 U/g) was highest at 30°C (Table 2). Since the fungus was mesophilic in nature, variation in temperature above or below its optimum (30°C) led to a decline in enzyme production. Likewise, maximum acid protease production was supported in *Rhizopus oligosporus* HIS_{13} at 30°C [27].

Supplementation of carbon, salts and nitrogen sources

The solid substrates used for fermentation, being agro byproducts, might lack in nutrients and hence external supplementation might positively contribute to enzyme production [21]. As revealed from the graphs, none of the carbon supplements showed any positive influence on the enzyme production (Table 3).

Table 3. Effect of various chemical parameters on acid protease activity of *Aspergillus* sp

Chemical parameters		Protease activity (U*/mL)
Carbon sources	dextrose	255±5.0^{c}
	fructose	150±2.0^{e}
	sucrose	270±2.0^{b}
	Skim milk	255±5.0^{c}
	Maltose	280±3.0^{a}
	lactose	236±7.64^{d}
	Starch	250±4.0^{c}
	Wheat bran(control)	280±2.0^{a}
Nitrogen sources	peptone	280±2.0^{c}
	casein	310±3.0^{b}
	Soyameal	300±5.0^{b}
	gelatin	310±6.53^{ab}
	Yeast extract	310±2.0^{b}
	Albumin	250±3.06^{d}
	$(NH_4)_2SO_4$	280±5.0^{c}
	KNO_3	321±3.18^{a}
	$(NH_4)_2HPO_4$	253±3.35^{d}
	NH_4Cl	230±2.0^{e}
	Wheat bran(control)	280±2.0^{c}

*Values are mean ±standard error from 3 replicates in each group, Values not sharing a common superscript letter(s) differ significantly at p<0.05(DMRT), * One unit of activity was defined as the amount of enzyme producing a change of absorbency equivalent to 1 µg of tyrosine per min at pH 5.0 and 30°C.*

Supplementation of sugars like lactose and fructose repressed the enzyme production. The repression of enzyme synthesis by these sugars is attributed to the catabolite repression effect, in which presence of easily assimilable carbon sources restricts enzyme synthesis [14,24]. However, supplementation of organic nitrogen sources like casein, gelatin, soy meal, YE and inorganic N source like potassium nitrate showed profound increase in enzyme production, with gelatin showing almost 1.21 fold increase (Table 3). This observation is in accordance with many reports that a nitrogen- rich media favoured protease production [19,28]. Tramacoldi *et. al.* (2004) recorded the highest acid proteolytic activity (80 U/ml) of *Aspergillus clavatus* in culture medium containing glucose and gelatin (1% w/v) at 30°C at the third day of incubation. However contrasting observations have been reported by Sumantha *et. al.*, (2005) [21], in which *A. oryzae* protease production did not show any positive influence on supplementation of either organic or inorganic nitrogen sources along with wheat bran. Neither did salt supplementation along with wheat bran show any profound influence on protease production

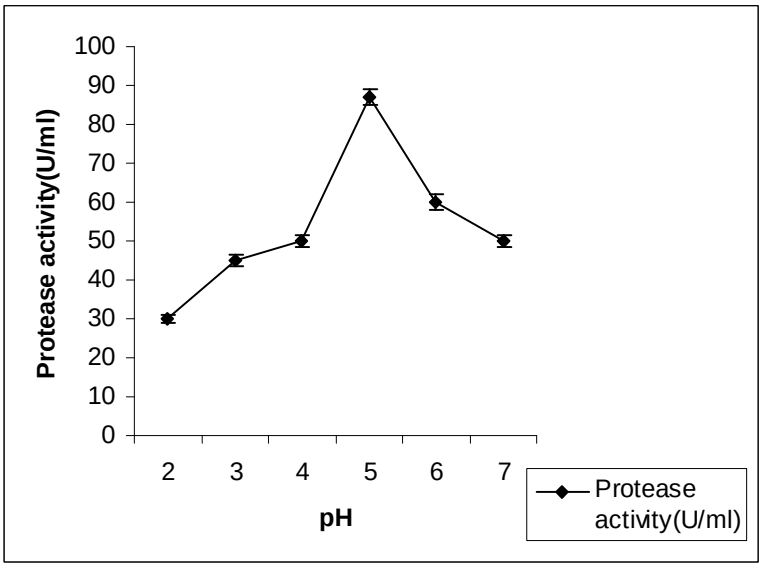
(data not shown). Optimum growth and acid protease production (340 U/g) by *Aspergillus* sp. was achieved with wheat bran supplemented with gelatin (1%, w/v) at pH 5.0, 30°C by 72 h.

Characterization of the Enzyme

Optimum pH

Preliminary studies to characterize the crude acid protease enzyme of *Aspergillus niger* used in the study revealed that the enzyme showed a gradual increase in activity and reached a peak at pH 5.0 which was then followed by a sharp decline as shown in Fig. 1 indicating the enzyme's instability at any pH other than its optimum, viz.5.0. The enzyme of *Monascus* sp.3403 was active in acid regions with the maximum at about pH 3.0 [30] which was similar to other fungal proteases (*Aspergillus* enzyme, pH 2.5 ~ 3.08} [10,29]; and *Rhizopus* enzyme [31]). The purified enzyme of *Rhizopus oryzae* showed a pH optimum of 5.5 [32]. The acid protease from *Mucor rouxii* showed optimum pH of 4.0 [33].

Fig.1. pH profile of the crude acid protease of *Aspergillus* sp



The crude enzyme showed highest activity at a temperature range of 50-60°C. The enzyme activity gradually increased with increasing temperature, followed by a steep decrease at temperatures above 60°C (Fig.2). The enzyme was stable for 30 mins at 50°C retaining almost 80 % activity (Fig.3).

Thermostability study of this enzyme at 50°C shows potential for applications in food industries such as baking, brewing etc. The acid protease of *Monascus* sp. 3403 showed a similar temperature optima of 55°C and was stable at 50°C for 10 mins [30]. The enzyme of *Rhizopus oryzae* acted optimally at 60 °C with activation energy of 15.16 kcal/mol and was stable in the temperature range of 30–45 °C [32]. The acid protease of *Mucor rouxii* showed temperature optima of 50°C and was inactivated at 70°C [33]. Tremacoldi *et. al.*, (2004) [29] reported temperature optima of 40°C by acid protease of *Aspergillus clavatus* with the half-lives at 40, 45 and 50 °C to be 30, 10 and 5 min, respectively.

Fig.2. Temperature profile of the crude acid protease of *Aspergillus* sp.

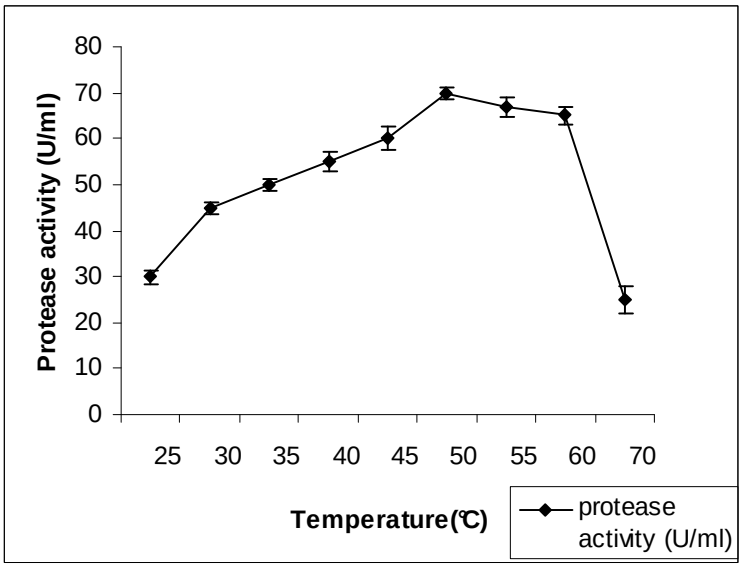
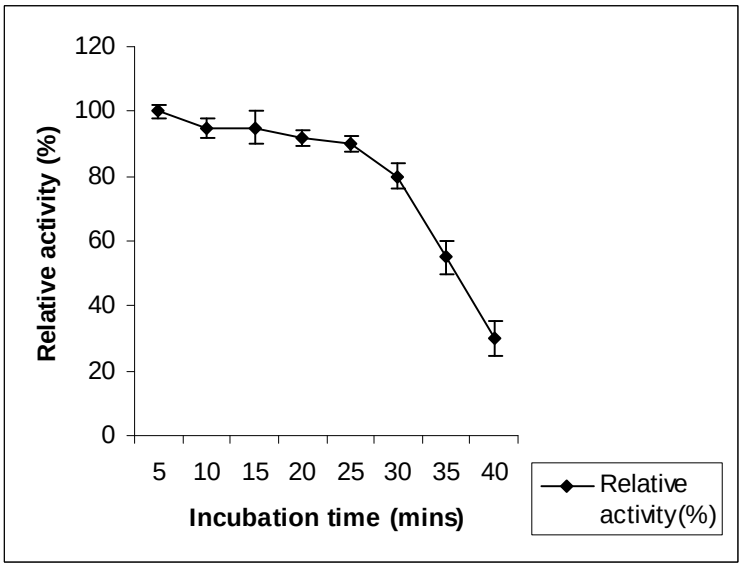


Fig.3. Stability profile of crude acid protease of *Aspergillus* sp. at its optimum temperature



Effect of metal ions

The study of the effect of the various metal ions on acid protease activity by *A.niger* indicates that the enzyme is a metalloprotease, since, Ca^{2+} and Mg^{2+} ions showed positive effect on acid protease activity, while additions of $Fe^{3+,}$ Cu^{2+} and Zn^{2+} showed a decrease in the activity as compared to the control, indicating possible inhibitory effect of these ions on the enzyme (Fig.4). However, studies need to be done with purified enzymes to confirm the same. The enzyme activity of acid protease from *Monascus* sp.3403 was not affected by metal ions like Hg^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+} [30].

Fig.4. Effect of metal ions on crude acid protease of *Aspergillus* sp

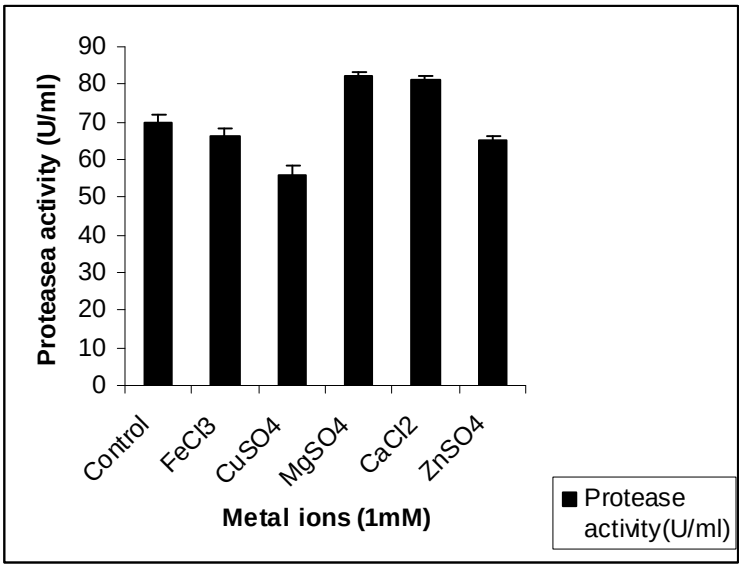
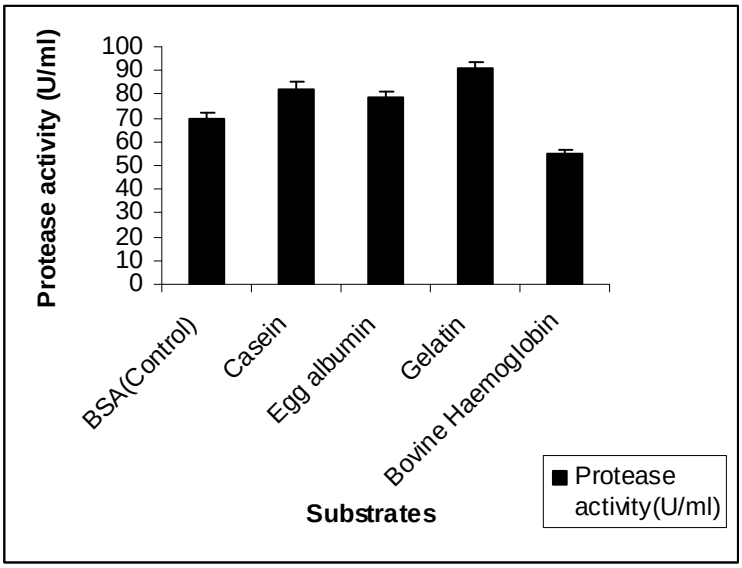


Fig.5. Effect of different substrates on protease activity.



Substrate specificity

When assayed with native protein as substrates, the protease showed high level of hydrolytic activity with all the substrates tested with highest specificity for gelatin (130 %), indicating broad substrate specificity of this enzyme (Fig.5). The enzyme also showed activity with Bovine haemoglobin at pH 2.5, confirming the acidic nature of the enzyme. These results are in agreement with the reports on fungi like *Rhizopus oligosporus* NRRL 2710, *Mucor dispersus* NRRL 3103 and *A.elegans* NRRL 3104 which were studied for their acid protease production and their role in soybean food fermentation [26].

CONCLUSION

Due to the irregular production associated with proteases and large number of moral and ethical issues, interest has been growing in microbial proteases- acidic, neutral and alkaline which have prominent commercial importance. Microorganisms, especially fungi like *Aspergillus*, *Rhizopus* and *Penicillium* which are potent strains for protease owing to their GRAS (Generally Regarded As Safe) nature, have now become popular, especially with respect to enzyme application in the food industries [34,35]. Enzyme production with solid state cultivation is more economical and convenient as compared to submerged fermentation for the utilization of agro-industrial byproducts. Hence it is feasible to apply solid state cultivation as an economic alternative in producing value added products and agricultural chemicals during the utilization and treatment of agricultural byproducts. In this study SSF of agro-industrial byproducts has been carried out with a potential acid protease producing fungal strain. Further optimization studies in this direction with a thorough understanding of the enzyme will definitely make this strain a better industrially viable option.

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