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PROTEASE PRODUCTION BY *Humicola grisea* THROUGH SSF

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ABSTRACT
Microbial proteases are the most important industrial enzymes with considerable applications in food, medicine and pharmacy. Fungi elaborate a wide variety of proteolytic enzymes than bacteria and also offer an advantage as the mycelium can be grown on cheaper substrate, broad range of pH and the mycelium can also be easily removed from the final product. Hence the present study was aimed to study the protease produced by a thermophilic fungus *Humicola grisea*, isolated from the cow dung. The protease production was evaluated in solid state fermentation (SSF) with different substrates and also under the influence of different activators and inhibitors. The most satisfactory results were obtained when wheat bran was used as substrate in SSF. The enzyme activity was also studied under immobilization for all the parameters.

KEY WORDS: Thermophile, *Humicola grisea*, Protease, solid state fermentation (SSF), wheat bran, cow dung.

INTRODUCTION

Proteases are enzymes that break down protein molecule through peptide bond hydrolysis (Eskin,*et.al,* 1971). The molecular weight of protease range from 18-90 KDA (Lester and Sidney, 1972). Proteases are commercially employed in many industrial processes. In foods, proteases have main application i.e. processing of new protein-based ingredients (Nagodawithana and Reed, 1993;). Proteases are also used in Pharmaceutical, waste management, meat processing, dairy, detergent and leather industries. Currently, microbial proteases make up approximately 40% of total world enzyme sale (Gupta *et al.,* 2002 and Deshpande *et al.,* 1998). Proteases are derived from microorganisms such as bacteria, fungi and yeast has found wide spread application in many fields (Byung *et al.,* 2008;). Fungi elaborate a wide variety of proteolytic enzymes than bacteria. The filamentous fungi have a potential to grow under various environmental conditions of time, pH and temperature, utilizing a wide variety of substrates as nutrients (Ikram *et al.* 2006). Easy immobilization of mycelium for separated use, spore substrate specificity and lastly low cost of production (Madhava and Pushpa, 2010). For the production of enzymes for industrial use, isolation and characterization of new promising strain is a continuous process (Balasubramanyan *et al.,* 2002). These are generally produced by solid state fermentation, a superior technique for the production of enzymes (Nigam *et al.,* 2001). Solid state fermentation involve microbial modification of a solid, un dissolved substrate in which microbial cultures are grown on moist solid with little or no free water, although capillary water may be present (Mudgett 2001).The product can be recovered in highly concentrated form as compared to those obtained by submerged fermentations. These not only provide a natural substrate for fungal growth and fermentation, but also result in improved value of these agro-industrial residues (Mitchell *et al.,* 2000). The environmental conditions of the fermentation medium play a vital role in the growth and metabolic production of microbial population. The pH of fermentation medium was reported to have substantial effect on production of proteases (Al.Shehri 2004). Thermophilic fungi are known to produce thermo stable enzymes. Besides thermal stability, these enzymes also exhibit higher stability towards other protein denaturating conditions such as extreme pH values and compounds such as ionic detergents and organic solvents, when compared to similar mesophilic enzymes (Gusek and Kinsella, 1988). Hence an attempt was made to isolate thermophilic fungi *Humicola grisea* from cow dung for the production of protease under solid state fermentation. The production was studied with different substrates and also under the influence of different activators and inhibitors.

MATERIALS & METHODS

Isolation of fungi

The fungus was isolated from cow dung, collected from different areas of Guntur and East Godavari. The collected sample was incubated for 24 hrs at 45-55^{0}c. The incubated sample was subjected for serial dilution, and then plated on Yeast-starch agar media reported by Cooney and Emerson (1964). By dilution pour plate method and incubated at 45-55^{0}c for the growth of mycoflora. The fungus was identified by using microbial atlas (Alexopolus and Mims 1979). This fungus was transferred to slants of Yeast-starch agar and was stored in refrigerator at 40^{0}c for further use.

Substrates and diluents

In the present study, Wheat bran was used as a substrate. Along with wheat bran, sesame oilcake, ground nut oil cake, cotton seed oil cake were selected and used as substrates. The composition of moistening agents (mineral media) include (%) 0.5 NH_4NO_3, 0.2 KH_2PO_4, 0.2 $MgSO_4$, 0.1 NaCl in water.

Production of Protease using wheat bran as substrate

The wheat bran was used as substrate for the production and optimization studies of protease.10gms of wheat brawn was mixed with 15 ml of moistening agent after sterilization and cooled to room temperature. The slants of

5–7 days old culture was wetted by adding 10 ml of distilled water; a homogeneous suspension was obtained by shaking for approximately 1 min. 1 ml spore suspension was used for inoculation and incubated for about 72 hrs in an incubator under room temperature. Protease activity was measured at different time intervals.

Preparation of enzyme extract

A 250 ml conical flasks containing 10 g of substrates with 15 ml of moistening agent were sterilized at 121^0C (15 1bs/inch pressure), cooled, inoculated and incubated at 30^0C. After incubation, 80 ml of distilled water was added to the culture flask, flask was shaken for 14 hrs at 200 rpm. The content of flask was filtered and filtrate was analyzed for enzymatic activity.

Protease Assay

The Protease activity in the crude enzyme extract was assayed by using 1% casein in citrate buffer (pH 7). The reaction mixture contains 1ml casein and 1ml crude enzyme extract and allowed to stand for 1hr at the room temperature. After 1hr, 5 ml trichloroaceticacid (TCA) solution was added to stop the enzymatic reaction. After addition of the TCA, the tubes were shaken and then contents were centrifuged at10, 000 rpm for 15mins for the sedimentation of the pellet. The supernatant was collected from the centrifugal tubes, and to this supernatant 5ml of NaOH solution was added and allowed to stand for another 15mins. Finally 0.5ml of FC reagent was added and the intensity of blue colour was measured at 700 nm within half an hour. One unit of enzyme activity was defined as the amount of enzyme released 1µg of tyrosine mL–1 of crude enzyme per hour.

Optimization of media parameters and substrate concentration:

Production of protease from *Humicola grisea* was optimized by controlling different physical and Chemical parameters like carbon source, nitrogen source and other components in the medium like$MgSO_4$, KCl, KH_2PO_4, pH range and temperature for the maximum yield of enzyme. The optimization experiments were conducted uniformly by varying one compound at a time and keeping the other conditions constant. Optimization studies were carried out at 24hrs, 48hrs and 72hrs. Wheat bran at different concentrations (2.5 to 12.5%) moistened with mineral medium was used for the determination of optimum concentration of substrate for the better production of protease.

Optimization of Nitrogen source, NaCl, KH_2PO_4 and $MgSO_4$

KH_2PO_4, $MgSO_4$ were optimized by taking at different concentrations (0.1 to 0.3%) for the better production of protease. To determine the optimum concentration of NaCl, different concentrations of NaCl between 0.025% to 0.125% were taken and fermentations were carried out. Ammonium nitrate was the nitrogen source in the medium. To determine the optimum concentration of NH_4NO_3 varied concentrations of NH_4NO_3 were taken from 0.3 to 0.7%. After incubation protease activity was studied.

Optimization of Temperature and pH

One of the most important factors is the incubation temperature, which is important for the production of proteases by microorganisms. For temperature optimization, different temperatures ranging from 35ºC to 75°C were taken and the activity of protease was studied. pH can effect growth of microorganisms either directly or indirectly by affecting the availability of nutrients or directly by action on cell surfaces. The metabolic activities of microorganisms are sensitive to pH changes and the pH of culture media has marked effect on the type and amount of enzyme produced. Changes in pH may also cause denaturation of enzyme resulting in the loss of catalytic activity (Anwari *et al.,* 2006). In the present study, the pH range for the optimization of pH selected varies from 6.5 to 8.5.

Immobilization of crude enzyme and its activity studies

Preparation of beads and enzyme immobilization:

The crude fungal protease was immobilized with sodium alginate and calcium chloride reported by Chellapandi (2007). A 2% sodium alginate solution was prepared by dissolving sodium alginate in 100 mL hot water. The contents were stirred vigorously for 10 min to obtain thick uniform slurry without any undissolved lumps. The crude fungal protease enzyme solution was mixed with sodium alginate solution (2%) in 1: 1 ratio.The protease-alginate mixture was added drop wise into calcium chloride (0.3M) solution from 0.5 cm height and kept for curing at 4^0C for 1 hr. The cured beads were recovered by filtration and thoroughly washed with distilled water and finally with 25mM tris-HCl buffer of pH 8.0. The enzyme assay was done using the same protocol mentioned above except replacing enzyme with enzyme entrapped beads.

RESULT & DISCUSSION

Microbial proteases have a number of commercial applications in industries like food, leather, meat processing and cheese making. A major commercial use is the addition of microbial proteases to domestic detergents for the digestion of pertinacious stains of fabrics (Nath *et al.,* 1980). It has been reported that the production of extracellular proteases by different microorganisms can be strongly influenced by the culture conditions. So, it becomes necessary to understand the nature of proteases and their catalytic potentiality under different conditions.

Production of protease using wheat bran (screening of fungi for the production of protease)

The protease produced from the isolated fungi, *Humicola grisea* successfully using wheat bran as substrate. 10% of the substrates have given a maximum activity of 961.25 IU after 72 hrs (Fig. 1) of incubation after which the production has declined. Immobilized enzyme had given max.activity 1263 IU at 7.5% conc. of wheat bran after two washes.

Optimization of Nitrogen source (NH_4 No_3)

The nitrogen source was optimized for the better production. 0.3% to 0.7% concentrations of the NH_4NO_3 had given maximum activity of 850 IU at 0.5% concentration (Fig. 2) after 72 hrs. Immobilized enzyme had given max.activity of 1067 IU at same concentration after two washes.

Optimization of KH_2PO_4, $MgSo_4$ and NaCl

Mineral media components viz. KH_2PO_4, $MgSo_4$ and NaCl were optimized for better production. 0.10, 0.15, 0.20, 0.25 and 0.30% concentrations of the KH_2PO_4 had given max. activity of 878.25IU at 0.20 concentrations (Fig. 3)

after 72 hrs and the immobilized enzyme had given 1068IU activity at same conc. after two washes. With the same concentrations of the Mgso4 had also given max.activity of 855 IU at 0.20 conc.(Fig. 4) after 72 hrs and immobilized enzyme had given 987IU at same conc. after two washes. 0.025, 0.050, 0.075, 0.100 and 0.125 concentrations of the NaCl had given max activity of 913 IU at 0.100 conc. (Fig. 5) after 72 hrs and at the same conc. immobilized enzyme had given 1110 IU activity after two washes. Certain nitrogenous salts tend to decrease the pH of the culture medium and had the adverse effect on enzyme production although they supported the growth of the organism (Lee and Wang 1996).

Effect of Inhibitors and Activators

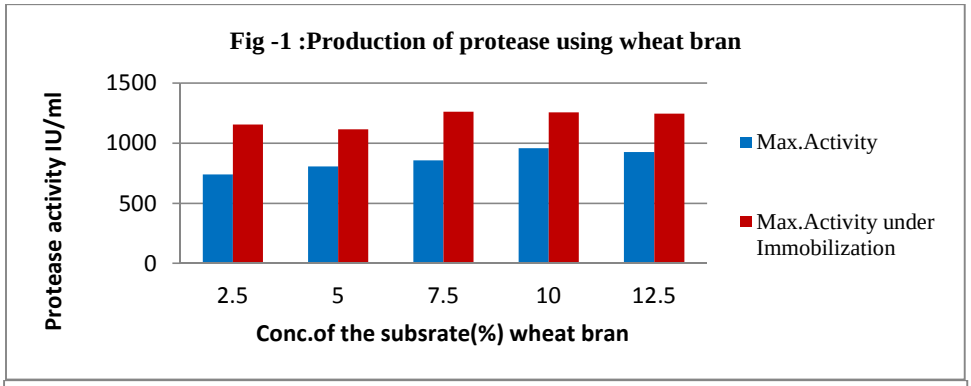
While studying the effect of activators it was observed that $ZnSO_4$ enhanced the protease activity up to 907.25 IU. 1038 IU of activity had given by immobilized enzyme after two washes followed by $FeSO_4$ and $CaCl_2$ (Fig. 6). In contrast, Nehra *et al.* (2002) reported thatMg^{2+} was found to be an activator of the alkaline protease enzyme produced by *Humicola grisea* suggesting these metal ions had a capability to protect enzyme against denaturation. The selected inhibitors were found to be less effective against the protease activity but the crude enzyme of *Humicola grisea* showed 50%inhibition in the presence of SDS indicating that the enzyme is an alkaline serine protease Work on serine protease has been reported by many researchers (Morihara 1974).

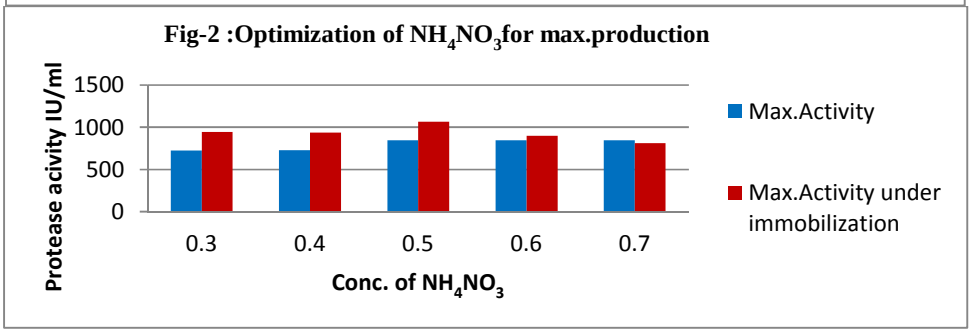
Effect of Temperature and pH

To further enhance the production of protease pH and temperature were also optimized. Maximum production was obtained at 65^0C and pH 8 (Fig. 7, Fig. 8). At the same levels immobilized enzyme had given max. activity 993 IU, 1036IU after two washes. Most alkaline proteases have been reported to have optimum activity in the range pH 8-9 (North 1982). Protease activity at 45^0C was also reported by Shumi *et al.* (2003) while working with the protease of *Fusarium tumidum.* Fungal proteases are usually thermo labile and show reduced activities at high temperatures (Tunga 1995). Higher temperature is found to have some adverse effects on metabolic activities of microorganism (Li *et al.*, 1997) and cause inhibition of the growth of the fungus. The enzyme is denatured by losing its catalytic properties at high temperature due to stretching and breaking of weak hydrogen bonds with in enzyme structure (Karan *et al.*, 1991). The maximum alkaline protease activity reported at 50°C isolated from *Thermomyces lanuginose* P134, and *Tritirachium albumlimber*.

Production by using different oil cakes

Among the selected oil seed cakes in place of wheat bran ,ground nut oil cake was found to be effective substrate (950.25) IU after wheat bran for the production of protease followed by Ground nut oilcake with *Humicola grisea* (Fig. 9). Immobilized protease had given max.activity of 1186 IU after two washes by Ground nut oil cake. In a study by Dabholkar and Shirish (2009) soya bean oil gave higher production followed by sesame oil cake. These results were in accordance with reported protease production in presence of different substrates (Adinarayana *et al.*, 2002); and different carbon sources have different influences on enzyme production by different strains. It might be due to the fact that increased level of substrate decreases the aeration and porosity of the medium, which were very essential for the proper growth of the organism.





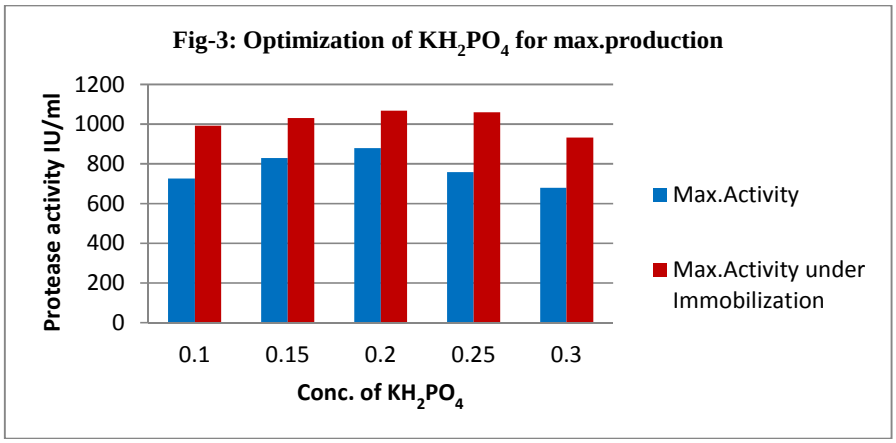


Fig-3: Optimization of KH_2PO_4 for max.production
Protease activity IU/ml
1200
1000
800
600
400
200
0
0.1
0.15
0.2
0.25
0.3
Conc. of KH_2PO_4
Max.Activity
Max.Activity under Immobilization

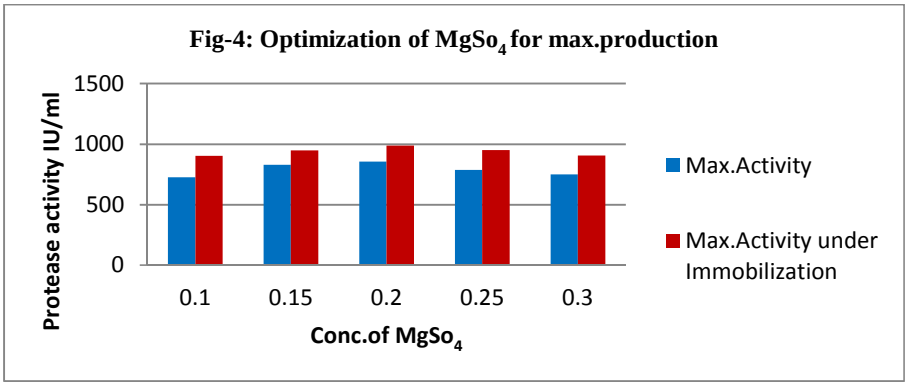


Fig-4: Optimization of $MgSo_4$ for max.production
Protease activity IU/ml
1500
1000
500
0
0.1
0.15
0.2
0.25
0.3
Conc.of $MgSo_4$
Max.Activity
Max.Activity under Immobilization

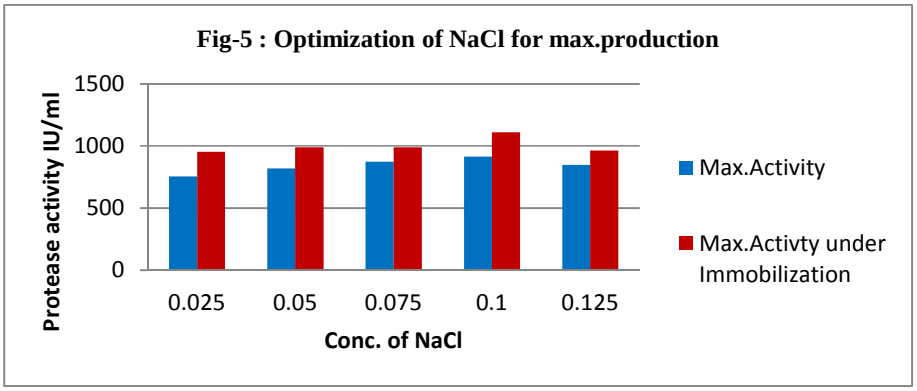


Fig-5 : Optimization of NaCl for max.production
Protease activity IU/ml
1500
1000
500
0
0.025
0.05
0.075
0.1
0.125
Conc. of NaCl
Max.Activity
Max.Activty under Immobilization

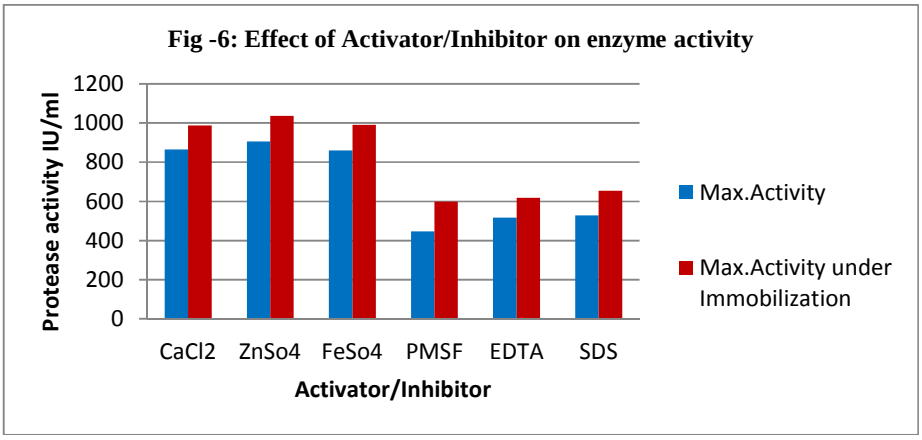
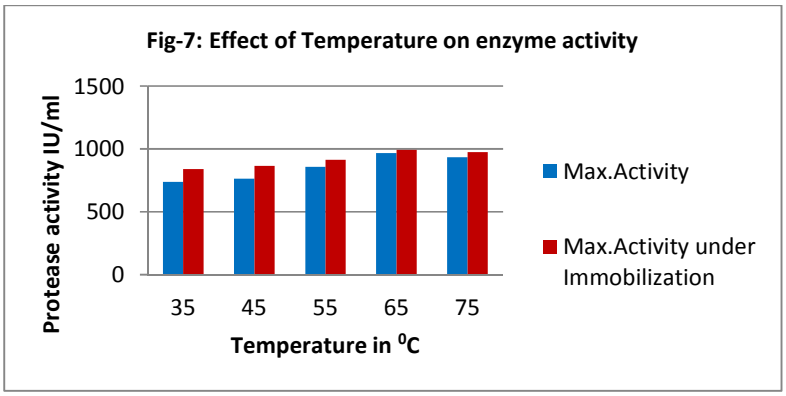
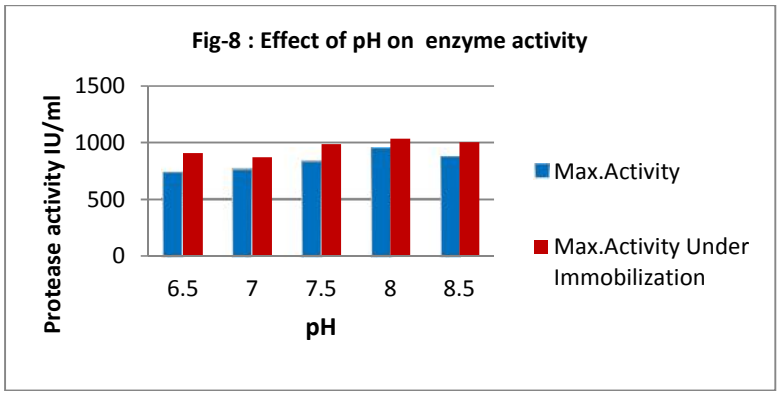
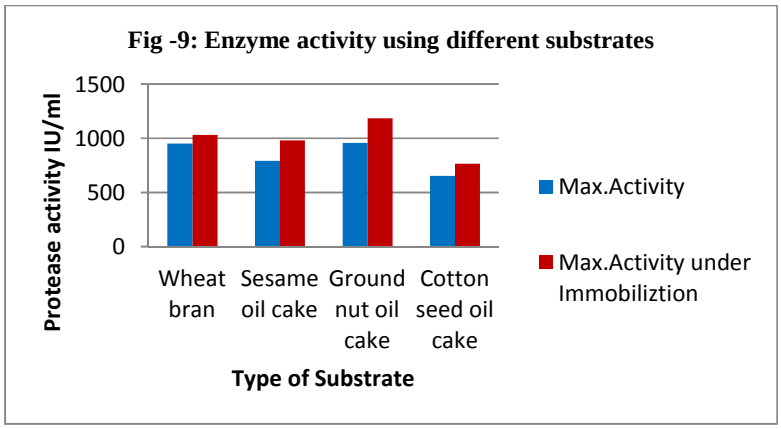


Fig -6: Effect of Activator/Inhibitor on enzyme activity
Protease activity IU/ml
1200
1000
800
600
400
200
0
CaCl2
ZnSo4
FeSo4
PMSF
EDTA
SDS
Activator/Inhibitor
Max.Activity
Max.Activity under Immobilization







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