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Production of Acid Protease by *Aspergillus niger* Using Solid State Fermentation

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Abstract.- The study is concerned with the isolation and screening of *Aspergillus niger* strains for the biosynthesis of acid protease using solid state fermentation. Thirteen *A. niger* strains capable of producing proteases were isolated from the soil. Of all the isolates, *Aspergillus niger* IHG_9 was found to be the best producer of protease. Different agricultural by-products were evaluated as fermentation substrate and maximum enzyme biosynthesis (5.2U/g) was obtained when sunflower meal was used as a substrate. Optimum temperature, pH and fermentation period for the production of protease were 30°C (7.2 U/g), 4.5(7.0 U/g) and 72 hrs (7.2 U/g), respectively. The growth and production of protease by *Aspergillus niger* IHG_9 was also studied by varying the carbon and nitrogen sources. Fructose (7.8 U/g) and beef extract (8.5 U/g) were found as best carbon and nitrogen sources, respectively. A spore inoculum at a level of 10% was found best for protease production (8.6U/g) by *Aspergillus niger* IGH_9 under solid state fermentation conditions.

Key words: Agricultural by-products, carbon source, mould, nitrogen source, proteolytic enzymes, solid state fermentation, *Aspergillus niger*.

INTRODUCTION

Proteolytic enzymes are included in a sub-class of the enzymes hydrolases. These enzymes cause breakdown of proteins into smaller peptides and amino acids by catalyzing the break down of peptide bonds. Proteases refer not to a single enzyme but to a mixture of enzymes which include proteinases, peptidases and amidases. The proteinases hydrolyze intact protein molecules to proteoses, peptones and some amino acids. Peptidases hydrolyze peptones to amino acids while amidases hydrolyze amino acids and release ammonia.

Proteolytic enzymes are the most important industrial enzymes, representing world wide sales of about 60% of the total enzyme market (Woods *et al.*, 2001). They are high temperature resistant with high specific activities and superior physical and chemical characteristics which seem to be good for future biotechnological applications, that is why they have wide applications in a large number of industrial processes (Rao *et al*., 1998; Temiz *et al*., 2008).

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The sources of proteases are plants, animal tissues and microorganisms but due to the low production of proteases from above sources, they are mainly produced by microorganisms which involve fungi, bacteria and some other microorganisms (West and Westport, 1983). The enzymes of fungi are largely acid proteases however; many fungi have also been reported to produce neutral and alkaline proteases (Tremacoldi *et al.*, 2004). Acid proteases are without doubt the most interesting group of proteases with respect to use in the food industry. They are characterized by maximum activity and stability at pH 2.0 to 5.0. Their molecular weight is around 35 kDa. The acid proteases are low in basic amino acid content and hence have low isoelectric points. They are insensitive to SH-reagents, metal chelators, heavy metals and DFP and are generally stable in the acid pH range (pH 2-6), but are rapidly inactivated at higher pH values. The acid proteases exhibit limited esterase activity but split a wide range of peptide bonds (Haq and Mukhtar, 2007).

Ever since it was found that extracts of *Aspergillus oryzae* and *A. flavus* possessed fibrinolytic (proteolytic) activity, many hundreds of fungal isolates have been screened for the enzyme which might be useful as the thrombolytic agent (Stefanini and Marin, 1982). Several investigations have revealed that the strains of different

Aspergillus species produce proteases (Barthomeuf *et al.*, 1988; Pourrat *et al.*, 1988; Battaglino *et al.*, 1991; Gomma *et al.*, 1989) but their production conditions and their activities are markedly varied. Acidic proteases are produced by several species of *Aspergillus* including *A. oryzae* (Narahara *et al.*, 1982), *A. niger* and *A. sojae* (Chakraborty *et al.*, 2000; Yang and Hsing, 1998). However some strains of *A. niger* (Heneri *et al.*, 1988) and *A. flavus* also produce alkaline protease (Malathi and Chakraborty, 1991).

For protease production from micro-organisms, both solid state and submerged fermentation techniques are employed (Pandey *et al.*, 2001; Mukhtar and Haq, 2008). However, solid-state fermentation is a preferred method for the production of acid proteases by the fungi (Tremacoldi *et al.*, 2004). The essential feature of solid-substrate fermentation (SSF) is the growth of micro-organisms on a pre-dominantly insoluble substrate without a free liquid phase. The substrates used in (SSF) are usually defatted oil seed cakes and other agricultural by-products. The moisture level in solid-state fermentation is the most important factor. It may be between 30 to 80% and for enzyme production is typically in the range of 60% (Tsuchiya *et al.*, 1998). The organisms which are most adapted to growth under these conditions of low water activity and presence of relatively intractable solid substrates are the fungi.

Pakistan being an agricultural country has a plenty of agricultural by products. So different agricultural by-products such as soybean meal, sunflower meal, rice bran, wheat bran, cottonseed meal and rapeseed meal have been evaluated for the biosynthesis of protease. Soybean meal was reported as a best agricultural by product for the production of protease from *Penicillium griseoroseum* (Haq *et al*., 2004). *Rhizopus oligosporous* was reported to produce protease using rice bran and sunflower meal as best agricultural by products (Ikasari and Mitchell, 1994; Haq *et al.*, 2003). Chakraborty *et al.* (2000) reported the production of protease from *A. niger* using wheat bran as a substrate.

The present study describes the production of protease from locally isolated *A. niger* by solid-state fermentation. Different cultural conditions were optimized for the maximum production of protease by *A. niger*.

MATERIALS AND METHODS

Isolation of micro-organism

The microorganism used in the present study was isolated from the soil of local habitat through serial dilution method using potato-dextrose-casein agar medium plates (PDA, 3.9g/L; casein, 1.0g/L and ampicillin, 100mg/L). The petriplates were incubated at 30°C for 3 days and the independent colonies forming larger haloes of casein hydrolysis on the isolation plates were picked up. The isolates were transferred on PDA slants and were stored in cooled cabinet at 4°C for further use. The isolated strains of *A. niger* were subjected to screening using solid state fermentation and the most potent strain showing highest protease production was selected for further use.

Inoculum preparation

In the present study, spore suspension was used to inoculate the fermentation flasks. Ten milliliters of sterilized 0.005% monoxal O.T. (Di-octyl ester of sulphosuccinic acid) was added to a 5 days old slant containing profuse growth of the microorganism and production of spores. The spores were scratched with the help of inoculating needle and the test tube was shaken to get homogenized spores suspension. One milliliter of the spores suspension was then transferred to the cotton plugged Erlenmeyer flasks containing fermentation substrate.

Fermentation procedure

Ten grams of fermentation substrate was moistened by adding 10ml of distilled water in 250 ml conical flasks. The flasks were plugged with cotton wool and were sterilized in an autoclave at 121°C (15lbs/inch^2 pressure) for 20 min. The flasks after cooling at room temperature were inoculated with 1ml of spore inoculum as prepared earlier. The flasks were then incubated at 30°C for 72 hours. The fermentation was run in triplicates and flasks were shaken twice, daily.

After 72 hours of incubation, 40ml of distilled water was added to each flask and these

were shaken on rotary shaker for one hour at 200 rpm. After that, the contents of flasks were filtered using Whattman filter paper No. 44 and the filtrate was used for enzyme assay.

Assay of protease

The activity of protease was assayed by the method of McDonald and Chen (1965) using casein as a substrate. To 1 m1 of the enzyme extract in the test tube, 4.0 ml of 1.0% casein was added. The enzyme sample was incubated at 35°C for one hour. The residual protein was precipitated by adding 5ml of 5% trichloroacetic acid. The precipitates were allowed to settle for 30 min and then centrifuged at 5000 rpm for 5 minutes. One milliliter of supernatant was mixed with 5 ml of alkaline reagent and 1ml of 1N NaOH. After 10 min, 0.5ml of Folin and Ciocaltaue reagent was added; as a result, blue color was produced. The optical density of the mixture was read at 700 nm on Spectrophotometer (Cecil-CE7200-series, Aquarius, UK)

One unit of protease activity is defined as the amount of enzyme required to produce an increase of 0.1 in optical density under defined conditions.

Effect of different substrates

Different agricultural by-products such as wheat bran, soybean meal, sunflower meal, rapeseed meal and cotton seed meal were evaluated for the production of protease in the present work. These substrates were procured from the local market and were ground and sieved to a uniform size.

Effect of different diluents

Different diluents were used to moisten the fermentation substrates in the flasks. The composition of the diluents was as follows:

D1: (g/l) $NaNO_3$, 2; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $ZnSO_4.7H_2O$, 0.1 and $FeSO_4.7H_2O$, 0.1.

D2: (g/l) Yeast extract, 10; Glucose, 10; Peptone, 10; $CaCO_3$, 5.0

D3: (g/l) Glucose, 10; Peptone, 10; Beef extract, 15 and NaCl, 5.0

D4: (g/l) $(NH_4)_2SO_4$, 1.5; Urea, 3.0; KH_2PO_4, 2.0, $MgSO_4.7H_2O$, 3.0 and $CaCl_2$, 3.0

D5: Distilled water

RESULTS AND DISCUSSION

Screening of isolates

The data of Table 1 shows the screening of isolates for the production of protease through solid-state fermentation. Thirteen different isolates belonging to *A. niger* were evaluated for the purpose. Of all the isolates tested, *A. niger* IHG_9 gave maximum production of proteases *i.e.*, 4.7 U/g. So *A. niger* IHG_9 was selected for further studies for the production of protease using solid state fermentation. All other isolates also showed considerable amount of protease production but less than the isolate IHG_9.

Table I.- Screening of *A. niger* isolates for protease production using solid state fermentation.

Isolates	Enzyme activity (U/g)
Aspergillus niger IHG_1	2.0±0.2
Aspergillus niger IHG_2	2.3±0.24
Aspergillus niger IHG_3	2.5±0.2
Aspergillus niger IHG_4	2.8±0.3
Aspergillus niger IHG_5	3.0±0.34
Aspergillus niger IHG_6	3.1±0.26
Aspergillus niger IHG_7	3.3±0.29
Aspergillus niger IHG_8	3.5±0.36
***Aspergillus niger* IHG_9**	**4.7±0.32**
Aspergillus niger IHG_{10}	3.7±0.38
Aspergillus niger IHG_{11}	3.8±0.26
Aspergillus niger IHG_{12}	4.2±0.3
Aspergillus niger IHG_{13}	4.0±0.28

Each value is average of three replicates; ± indicates the standard error from mean.
Incubation temperature, 30°C; Incubation period, 72 hrs; Substrate, wheat bran and soybean meal (1:1); Diluent, Distilled water.

Selection of substrate

Different agricultural by-products such as wheat bran, sunflower meal, soybean meal, cottonseed meal and rapeseed meal were evaluated for the biosynthesis of protease by *A. niger* IHG_9. Of all the by-products examined, sunflower meal gave maximum enzyme activity *i.e*., 5.2 U/g. Wheat bran, soybean meal, cottonseed meal and rapeseed meal gave 3.2, 4.8, 4.0 and 3.3 U/g of protease activity, respectively (Fig. 1). Sunflower meal gave maximum yield of protease because it may have adequate supply of proteins, carbohydrates and

minerals needed to the organism for growth and synthesis of proteases. Sunflower meal is the by-product of oil mills and is easily available in the market at very low price. Heneri *et al.* (1988) have also evaluated different protein substrates for the production of proteolytic enzyme by *A. niger* and found soybean meal as a best substrate.

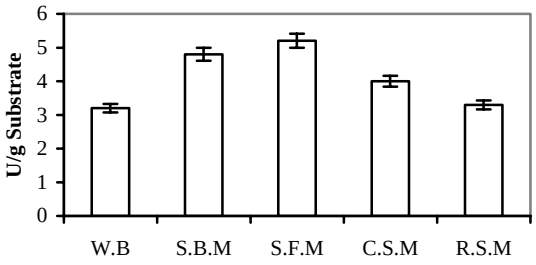

Fig 1. Screening of different agricultural by-products for the production of protease by *Aspergillus niger* IHG_9 using solid-state fermentation. Each value is a mean of three replicates. Y- error bars indicate the standard deviation from the mean value. W.B, wheat bran; S.B.M, soybean meal; S.F.M, sunflower meal; C.S.M, cotton seed meal; R.S.M, rape seed meal. Incubation temperature, 30°C; Incubation period, 72 hrs; diluent, distilled water.

Effect of diluents

The production of proteases by *A. niger* was investigated using different diluents for moistening the substrate. The production of enzyme was maximum *i.e.*, 6 U/g when substrate was moistened with diluent D_4. The diluent D_4 consisted of yeast extract, glucose, peptone and $CaCO_3$. Other diluents such as D_1, D_2, D_3, D_5 and D_6 gave enzyme production as 4.1, 3.3, 3.6, 3.2 and 3.8 U/g, respectively (Fig. 2). The enzyme synthesis was maximum when D_4 was used as diluent, which indicated that the organism required some additional nutrients for its optimum growth. So the deficient nutrients from the substrate were supplied by the diluent D_4 for growth of the organism and production of the enzyme. It also seems that the nutrients present in other diluents may not be sufficient or may have an inhibitory action on the growth of the organism and subsequently on the enzyme production, so gave less production of the enzyme (Battaglino *et al.*, 1991).

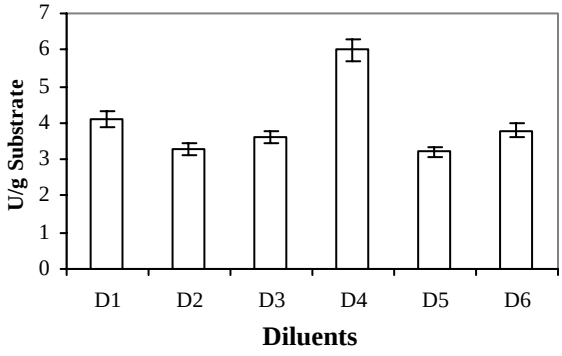

Fig. 2. Effect of different diluents on protease production by *Aspergillus niger* IHG_9 using solid -state fermentation. Each value is a mean of three replicates. Y- error bars indicate the standard deviation from the mean value. Incubation temperature, 30°C; Incubation period, 72 hrs; Substrate, wheat bran and sunflower meal (1:1).

Effect of pH of diluent

Figure 3 shows the effect of pH of diluent on the productivity of protease by *A. niger* IHG_9. The pH studied was ranged from 4.0 to 7.0 and the enzyme production was found to be maximum *i.e.*, 7.0 U/g when pH of the diluent was adjusted at 4.5. The pH has marked effect on the type and amount of enzyme produced by the microorganism. One organism may secrete variable amounts and types of enzymes depending upon the pH and composition of medium (Kubackova *et al.*, 1975). Changes in pH may also cause denaturation of enzyme resulting in loss of catalytic activity. It may also cause change in the ionic state of substrate which may result in the formation of charged particles which may not correspond with the ionic active sites of enzyme. Thus enzyme substrate complex will not be formed and the substrate will become unavailable to the microorganism. So, each enzyme acts best in a certain pH range which is peculiar to it and its growth and activities slow down with any appreciable decrease or increase in that value (Karuna and Ayyanna, 1993).

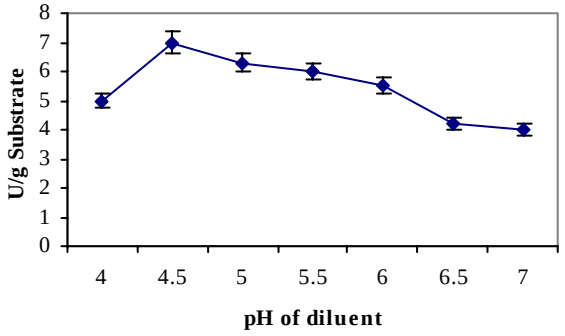

Fig. 3. Effect of pH of diluent on enzyme production by *Aspergillus niger* IHG_9 using solid -state fermentation. Each value is a mean of three replicates. Y- error bars indicate the standard deviation from the mean value. Incubation temperature, 30°C; Incubation period, 72 hrs; Substrate, wheat bran and sunflower meal (1:1); Diluent, D_4.

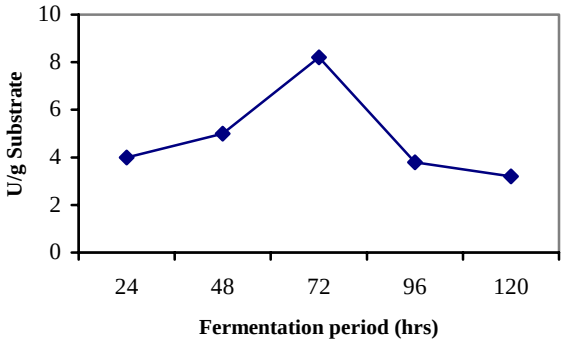

Fig. 4. Rate of fermentation for protease production by *Aspergillus niger* IHG_9 using - solid state fermentation. Each value is a mean of three replicates. Y- error bars indicate the standard deviation from the mean value. Incubation temperature, 30°C; Substrate, wheat bran and sunflower meal (1:1); Diluent, D_4.

Rate of fermentation

To determine the optimal incubation time for the production of protease by *A. niger* IHG_9, fermentation flasks were incubated for 24, 48, 72, 96 and 120 hours duration. The analysis of enzyme activity at different time intervals showed that maximum activity of protease *i.e.*, 7.2 U/g was achieved after 72 hours of incubation (Fig.4). After 72 hours, the enzyme activity was slightly declined but after 96 hrs, there was a sharp decline in the production of protease. In a similar study, Karuna and Ayyana (1993) reported that maximum protease activity with *A. oryzae* was obtained after 72 hours of incubation. When incubation period was increased up to 96 and 120 hours, enzyme activity was decreased. The incubation period is directly proportional to the production of enzymes and other metabolites up to a certain extent. After that, the enzyme production and growth of the microorganism starts to decline which can be attributed to the decrease in supply of nutrients to the microorganism and accumulation of toxic metabolites (Romero *et al.*, 1998).

Effect of incubation temperature

The fermentation flasks were incubated at different temperatures *i.e.*, 25, 30, 35, 40 and 45°C to study the effect of incubation temperature on protease production. The optimum temperature for enzyme production was found to be 30°C where maximum production of protease 7.2 U/g was observed (Fig. 5). As the temperature was increased above 30°C, the enzyme production was decreased.

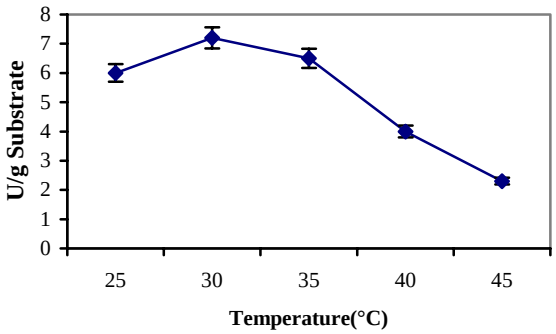

Fig. 5. Effect of temperature on enzyme production by *Aspergillus niger* IHG_9 using solid -state fermentation. Each value is a mean of three replicates. Y- error bars indicate the standard deviation from the mean value. Incubation period, 72 hrs; Substrate, wheat bran and sunflower meal (1:1); Diluent, D_4; pH of diluent, 4.5.

The rate of all the physiological processes is increased by increasing temperature but beyond certain limits it starts decreasing because enzymes

are sensitive to temperature. Rise of temperature within certain limits has the effect of increasing the activity of enzymes but very high temperatures are destructive. An enzyme loses its catalytic properties at high temperature due to stretching and final breaking of weak hydrogen bonds present in enzyme structure; this results in the complete change in the nature of the enzyme and makes them inactive (Conn *et al.*, 1987).

Effect of carbon sources

Effect of different carbon sources such as glucose, fructose, maltose, starch and sucrose was studied for the production of protease by *A. niger* IHG_9 (Fig. 6). Fructose gave maximum enzyme production *i.e.*, 7.8 U/g while glucose, maltose, starch and sucrose gave enzyme production as 6.5, 5.0 and 4.0 U/g, respectively. So the carbon requirement of the organism was fulfilled by fructose. The results were in accordance with those made by Sutar *et al*. (1990) who reported that fructose acted as a best carbon source for the production of alkaline protease from *Conidiobolus coronoatus*. It has been frequently described that in

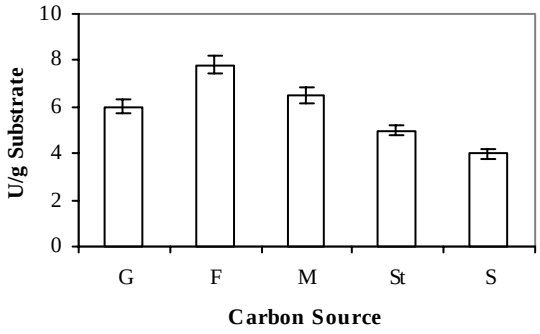

Fig. 6. Effect of carbon source on enzyme production by *Aspergillus niger* IHG_9 using solid -state fermentation. Each value is a mean of three replicates. Y- error bars indicate the standard deviation from the mean value. G, glucose; F, fructose; M, maltose; St, starch; S, sucrose. Incubation temperature, 30°C; Incubation period, 72 hrs; Substrate, wheat bran and sunflower meal (1:1); Diluent, D_4; pH of diluent, 4.5.

a defined medium, a protein source must be present for the enzyme to be produced. However, it has also been noticed that the absence of a proper carbohydrate (C-source) in the medium results in a dramatic decrease in enzyme production (Gajju *et al*., 1996), so a C-source is always an essential component of a fermentation medium.

Effect of nitrogen sources

Effect of different nitrogen sources such as urea, nutrient broth, meat extract, peptone and beef extract was studied on the production of protease by *A. niger*. Beef extract was found to be the best nitrogen source giving maximum enzyme production *i.e*., 8.5 U/g while urea, nutrient broth,

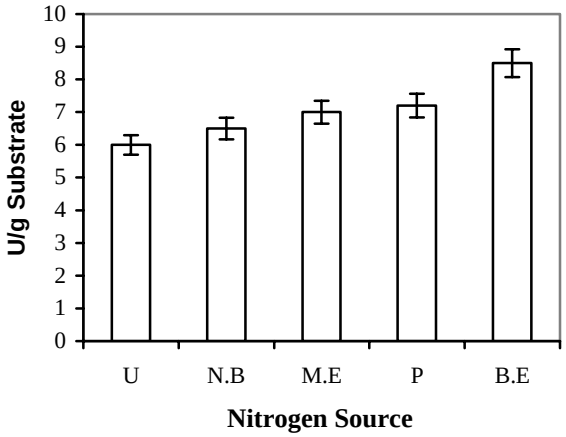

Fig. 7. Effect of nitrogen source on enzyme production by *Aspergillus niger* IHG_9 using solid -stat fermentation. Each value is a mean of three replicates. Y- error bars indicate the standard deviation from the mean value. U, urea; N.B, nutrient broth; M.E, meat extract; P, peptone; B.E, beef extract. Incubation temperature, 30°C; Incubation period, 72 hrs; Substrate, Wheat bran and sunflower meal (1:1); diluent, D_4; pH of diluent, 4.5

meat extract and peptone gave enzyme production as 6.0, 6.3, 7.0 and 7.2 U/g, respectively (Fig. 7). Nitrogen is very important in metabolism of microorganisms especially in the synthesis of enzymes and other proteins. So nitrogen has been always one of the important components of fermentation substrate/media. El-Zalaki *et al*. (1974) and Mukhtar and Haq (2003) used peptone as a best nitrogen source for the protease production by *A. niger* and *Rhizopus oligosporous,* respectively.

Effect of size of inoculum

Figure 8 shows the effect of the size of spore inoculum on the production of protease by *A. niger*. The size of inoculum was ranged from 5% to 20% (0.5ml to 2ml). The result showed that maximum amount of enzyme (8.6 U/g) was produced when 10% (1ml) of spore inoculum was added to the fermentation flasks.

The size of inoculum has great influence on the production of protease. An appropriate inoculum size is essential for optimum growth and enzyme production by the microorganism. Highest yield at 1.0ml of inoculum lies in the fact that a sufficient quantity of mycelium was formed, which produced optimum level growth of the mould and subsequently maximum production of enzyme. As the amount of mycelium was increased, it rapidly consumed majority of the substrate for growth purposes, hence enzyme synthesis was decreased (Carlile *et al*., 2001).

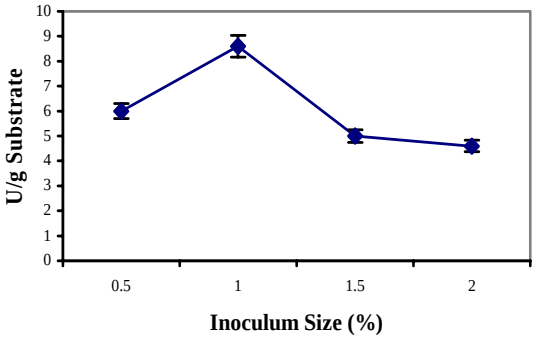

Fig. 8. Effect of size of inoculum on enzyme production by *Aspergillus niger* IHG_9 using solid-state fermentation. Each value is a mean of three replicates. Y- error bars indicate the standard deviation from the mean value. Incubation temperature, 30°C; Incubation period, 72hrs; Substrate, wheat bran and sunflower meal (1:1); diluent, D_4; pH of diluent, 4.5.

CONCLUSION

It is concluded from the present studies that the culture of *A. niger* IHG_9 was a good producer of acid proteases using sunflower meal as a substrate in solid state fermentation. It is also concluded that suitable carbon and nitrogen sources result in a significant rise in the protease production by the mould cultures.

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