Production of Poultry Enzyme, Protease from *Aspergillus niger*

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Abstract

In this study an industrial strain of *Aspergillus niger* is used for protease production. This strain of *A.niger* is maintained by following methods such as sub culturing and glycerol stocks. Protease production is through solid state fermentation, growing *A.niger* on agro-industrial waste such as wheat bran etc. This study includes the optimization of different parameters such as substrates, pH, temperature, moisture %, Diluents etc.

Solid state fermentation of *A.niger* on wheat bran produces maximum enzyme activity (1.210 O.D @660 nm corresponding to 179.86 Units/ml) which is a very high activity as compared to protease activity isolated from bacterial source. Efforts are being made to formulate protease from *A.niger* for the purpose of poultry feed supplement.

KEYWORDS: Poultry enzyme, Protease, *Aspergillus niger*, Fermentation

1. Introduction

Aspergillus niger is a standout amongst the most vital microorganisms in biotechnology. It has been now used to create extracellular enzymes, such as, glucose oxidase, pectinase, α-amylase and glucoamylase, organic acids, and recombinant proteins. Moreover, *A. niger* is utilized for biotransformations and waste treatment Roth and Dersch (2010), Schuster et al (2002), van Dijck et al (2003). Among the different enzymes created by the fungus are incorporated proteases. The major extracellular proteolytic activities in *A. niger* seem, by all accounts, to be because of acid proteases Jarai and Buxton (1994). Acid proteases are endopeptidases that rely upon aspartic acid deposits for their

reactant action and show maximal action at low pH. These enzymes offer an assortment of uses in the food, beverage industry, and medicines Vishwanatha et al (2009).

The Solid State Fermentation (SSF) is especially suitable for the fungi growth because their moisture requirements are lower compared to the bacteria. In this technique, the enzymes produced are more concentrated than those in submerged fermentation. SSF is an inexpensive technique and can be widely applied to agricultural products or by-products as substrates. Furthermore, the substrate must be easy to handle, inexpensive and easy to purchase. The overall cost of enzyme production is very high (due to high cost of substrates and mediums used). Therefore, development of novel processes to increase the yield of proteases with respect to their industrial requirements coupled with lowering down the production cost is highly appreciable from the commercial point of view.

2. Materials and Methods:

2. 1 Microorganism and inoculum development

Fungi: *Aspergillus niger*

The fungal culture was obtained from the culture bank and the culture was revived by transferring to the potato dextrose agar slants which were placed at the 35^0c for 24 hours. The culture was maintained by transferring into the new slants and stored in the cool laboratory at 4^0c.

2.2 Preparation of fungal inoculums:

Fungal inoculums were prepared in 500 ml conical flasks containing 100ml of sterilized potato dextrose broth and was aseptically inoculated with the *A.niger* from a fresh slant and allowed to grow at 35^0c for 48/72 hours in a incubator, after 48/72 hours of growth, the fungal culture was used as inoculums

2.3 Fermentation technique

Solid state fermentation was carried out for the production of protease by *A.niger*. Different types of substrates such as the wheat bran, soya meal, rice bran, rice husk, cotton meal, wheat + soya mixture etc., were used, these different types of substrates were taken in different amounts and to this different

amounts of different minerals and salt solution was added, and sterilized in an autoclave at 15 lbs pressure and 121^0c for about 15 minutes. After sterilization medium was cooled and different percentages (%) of microbial inoculums were inoculated to the substrates in the laminar air flow chamber in order to maintain the aseptic conditions, the substrates were mixed vigorously for uniform distribution in the medium and were incubated at 35^0c and room temperatures for different incubation periods.

2.4 Extraction of enzyme

After incubation period of SSF, different amounts of water/buffers was added to flask and the flasks were rotated on rotary shaker for one hour at a speed of 200rpm for the extraction of protease from fermented substrate, after shaking, the contents of flaks were centrifuged at 4^0C and 10000rpm. After centrifuge the supernatant (crude enzyme) was stored at 4^0C for further analysis.

2.5 Assay of protease

The activity of protease was assayed by the modified method of McDonald and Chen (1965). Two tubes were taken each with 5ml of casein and in one of the tube1ml of diluted enzyme was added and incubated at 37^0c for 10 min after incubation 5ml of TCA was added in both the tubes and 1ml of enzyme was added in other tube and incubated both the tubes at 37^0c for 30 min, after incubation the contents were filtered through the whattman filtered paper.2mL of filterate was mixed with 5mL of alkaline reagent (sodium carbonate) and to this 1ml of 1:1 diluted folin and ciocalteau reagent was added; as a result, blue colure was produced. The optical density of the test mixture was read against blank at 660nm on UV/VIS Spectrophotometer.

2.6 Standard Curve:

Standard curve was done by pipetting the following reagents into suitable tubes (in milliliters):

Table 1: preparation of tyrosine standard curve

Serial	Std soln (Tyrosine standard) 1.1mM	Distilled Water	Conc of tyrosine (µM)	Na_2CO_3 (0.5M)	F-C phenol reagent	O.D Values @660nm
Std Blank	-------	2	-------	5	1	0.000
Std 1	0.05	1.95	27.5	5	1	0.128
Std 2	0.10	1.90	55	5	1	0.252
Std 3	0.20	1.80	110	5	1	0.482
Std 4	0.30	1.70	165.1	5	1	0.725
Std5	0.40	1.60	220	5	1	0.910
Std6	0.50	1.50	275	5	1	1.117
Std7	0.60	1.40	330	5	1	1.321
Std8	0.70	1.30	385	5	1	1.507
Std9	0.80	1.20	440	5	1	1.764
Std10	0.90	1.10	495.4	5	1	1.854
Std11	1	1	550	5	1	2.102

Standard graph was drawn by placing O D values on X-axis and tyrosine concentration on Y-axis

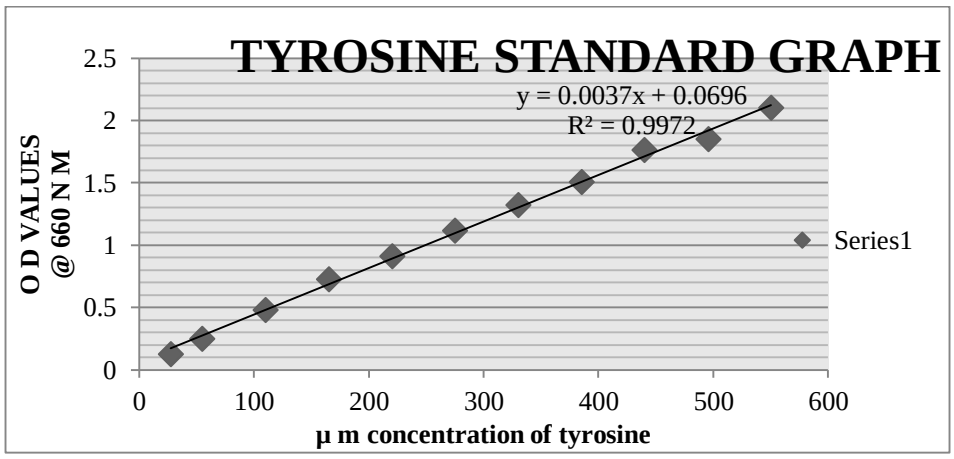

Fig no: **1 Tyrosine standard graphs**

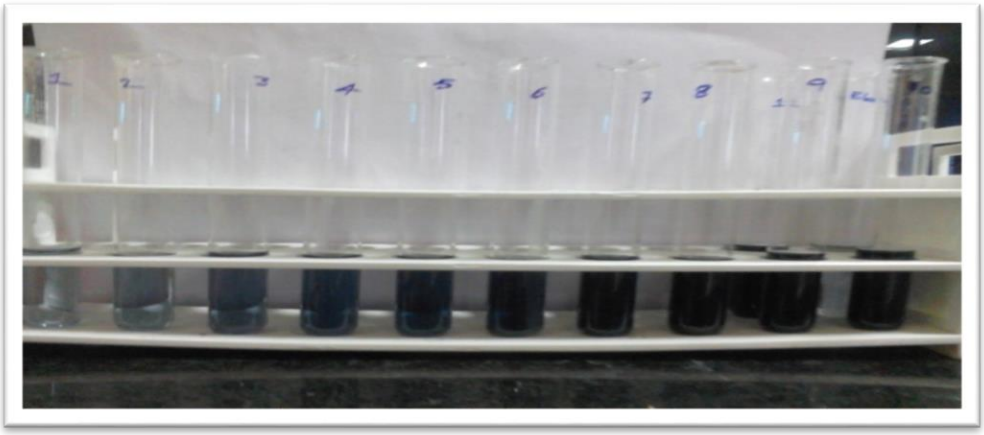

***Fig no*: 2 Tyrosine standard assay results showing concentrations gradient**

2.7 CALCULATIONS:

Standard Curve:

□A660nm Standard = A660nm Standard - A660nm Standard Blank

Plot the □A660nm Standard vs. µmoles of Tyrosine.

Sample Determination:

□A660nm Sample = A660nm Test - A660nm Sample Blank

Determine the µMoles of Tyrosine equivalents liberated using the Standard curve.

$$\text{Units/ml enzyme} = \frac{(\mu\text{Mole Tyrosine equivalents released})\ (11)}{(1)\ (10)\ (2)}$$

11 = Total volume (in milliliters) of assay

10 = Time of assay (in minutes) as per the Unit Definition

1 = Volume of enzyme (in milliliter) of enzyme used

2 = Volume (in milliliters) used in Colorimetric Determination

$$\text{Units/mg solid} = \frac{\text{Units/ml enzyme}}{\text{Mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{Units/ml enzyme}}{\text{Mg protein/ml enzyme}}$$

3. Results and Discussion

1) Effect of substrate:

Up to 40% of the total production cost of enzymes is due to the growth substrate. The real and beneficial production of enzyme is produced from the natural sources and industrial wastes. The

production medium costs of about 30-40% of the total production cost of the enzyme at industrial level. The production of protease was done by replacing the nitrogen sources and common substrates with Rice bran, Wheat bran, Soya meal, Cotton meal, Ground nut cake. The maximum production was occurred when Wheat bran used as natural substrate (179.86 U/ml). The result indicates that the wheat bran can also be used as the cheap substrate for protease production.

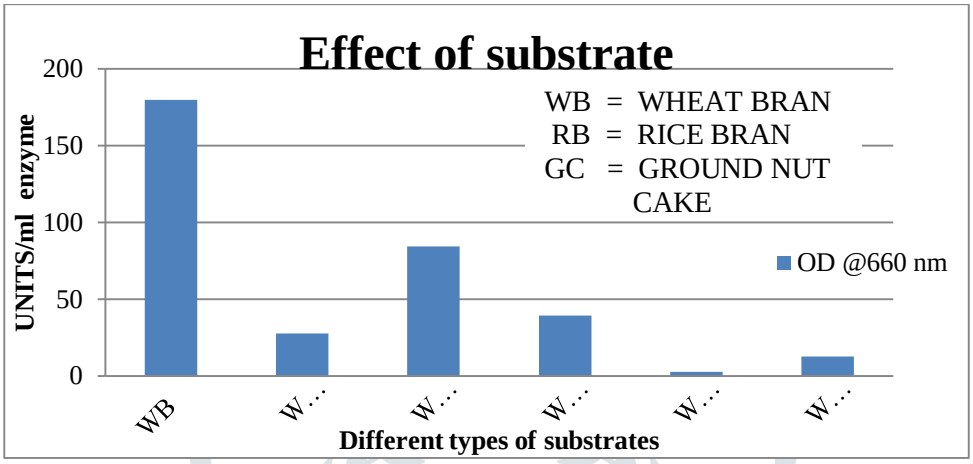

Fig no*: 3 Effect of substrate on protease production by *Aspergillus niger

2) Effect of diluents (mineral and salt solution)

Different diluents such as D2, D3, and D4 containing different nutrients were used for moistening the fermentation substrate. Maximum enzyme production was observed when D2 and D4 was used as diluents. The diluents D2 containing components had a positive influence on the productivity of protease. It indicates that the organism required additional nutrients for its growth. All the deficient nutrients from the substrate were supplied by diluents D2 and D4 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 it had less production of the enzyme.

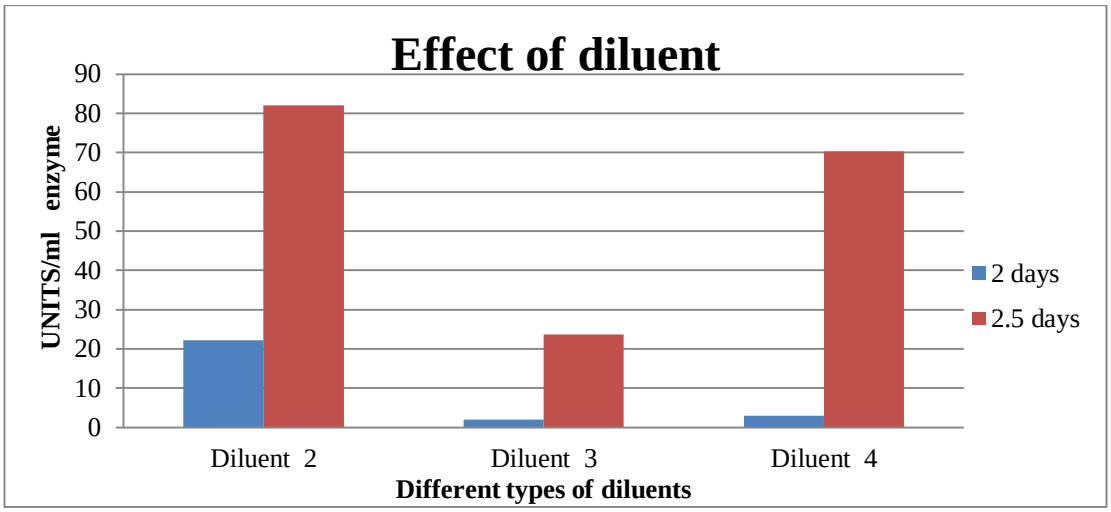

Fig no* 4: Effect of diluents on protease production by *Aspergillus niger

3) **Effect of Calcium chloride (**$CaCl_2$**)**

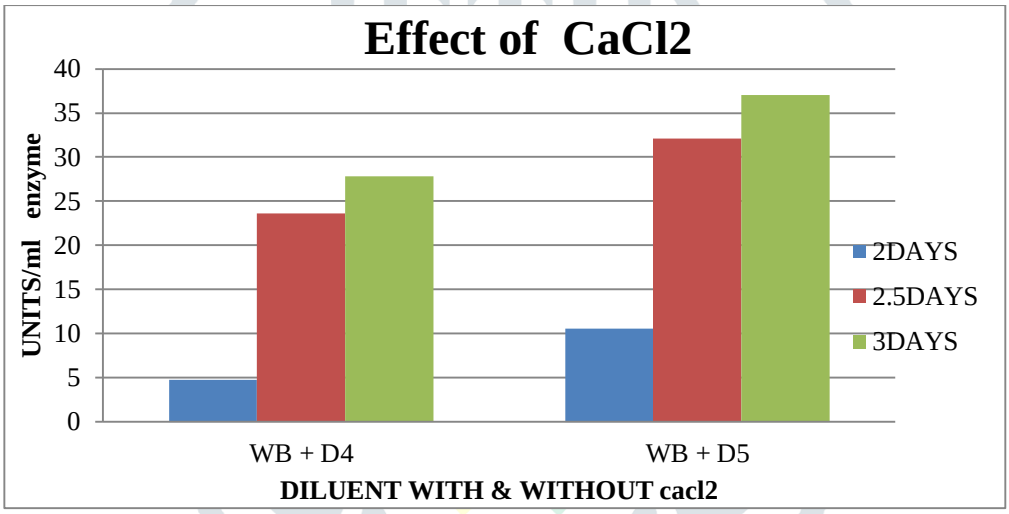

Fig no* 4: Effect of CaCl2 on protease production by *Aspergillus niger

4) Effect of incubation period:

Fermentation experiments for protease production under solid state fermentation were carried out for different time intervals ranging from 2days to 4days. After 48 h of incubation, the amount of protease production was 10.55 U/ml and it was increased with increase in the incubation period and reached maximum (37 U/ml) after 4days of incubation. Further increase in the incubation period resulted in the decreased enzyme production.

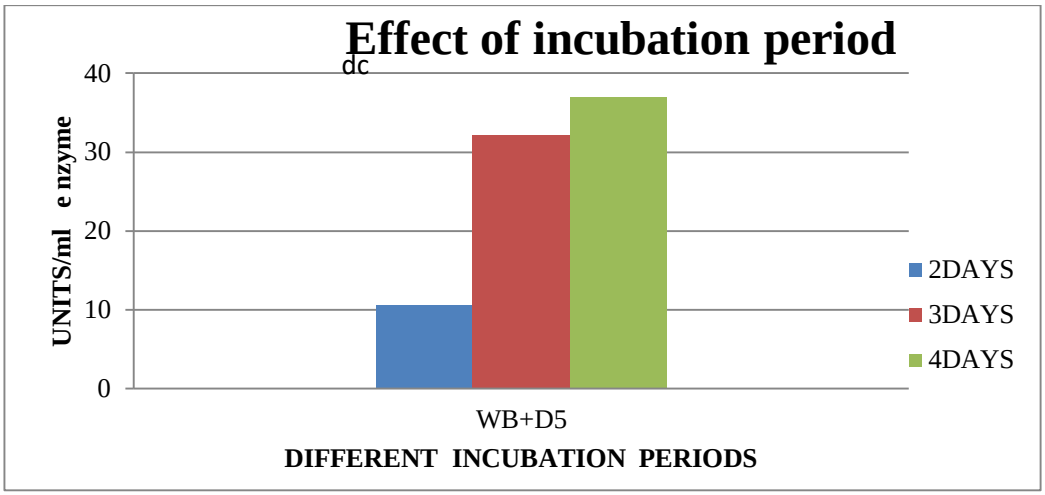

Fig no:* 5: effect of incubation time for protease production by *Aspergillus niger

5) Effect of temperature:

Effect of temperature on enzyme production by the solid substrate fermentation using *A.niger.* SSF with common in their substrates, diluents (D5), diluents amount (150%), inoculums (10%), incubation temperature (35^0c) are incubated for different incubation periods at different temperature conditions such as at 35ºC and at room temperature.

Temperature has the great effect on the solid state fermentation as from the graph it is cleared that at 35 temperatures incubated SSF has showed the higher results i;e (179 units/ml).

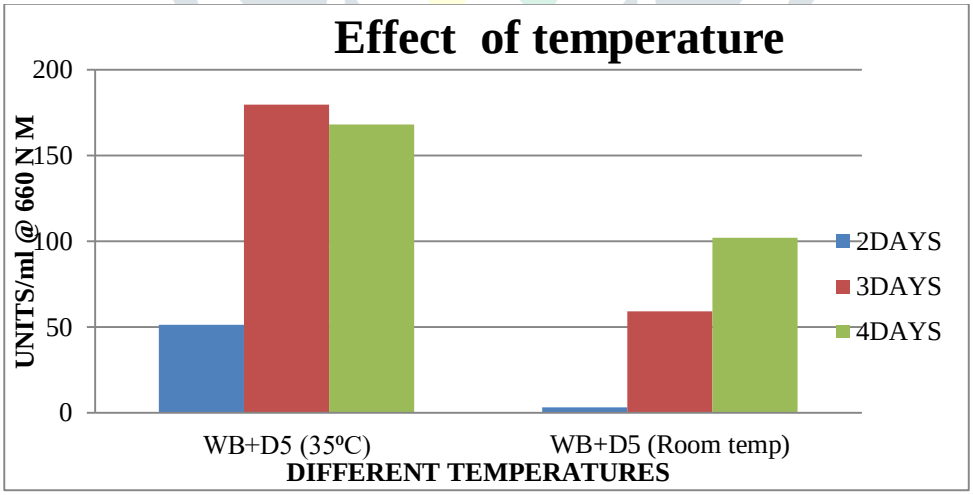

Fig no:* 6: Effect of temperature on protease production by *Aspergillus niger

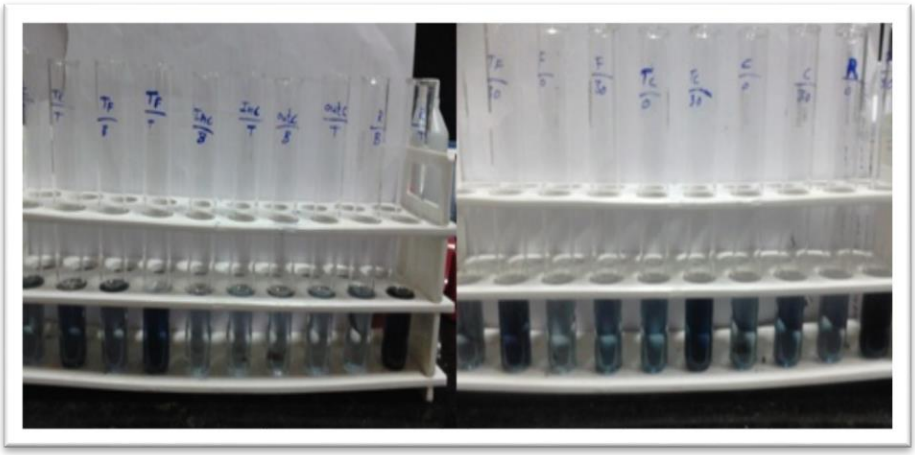

***Fig no* 7: Protease assay of crude enzyme of SSF at different temperature**

6) Effect of substrate particle size:

Substrate particle size is also one of the parameter which has the relative effects on the production of the enzyme by the solid substrate fermentation. Generally, smaller substrate particles provide larger surface area for microbial attack and, thus, are a desirable factor. Using the different particle sizes of the similar substrate having common in their substrate amount ,diluents type ,diluents amount, inoculums size, inoculums age, incubation period ,incubation temperatures, similar extraction has showed the following results mentioned in the below table. Substrate particle size i.e. (wheat bran) with fine quality and mixed (coarse & superfine) quality has the showed good results when compared with the other quality of the substrates.

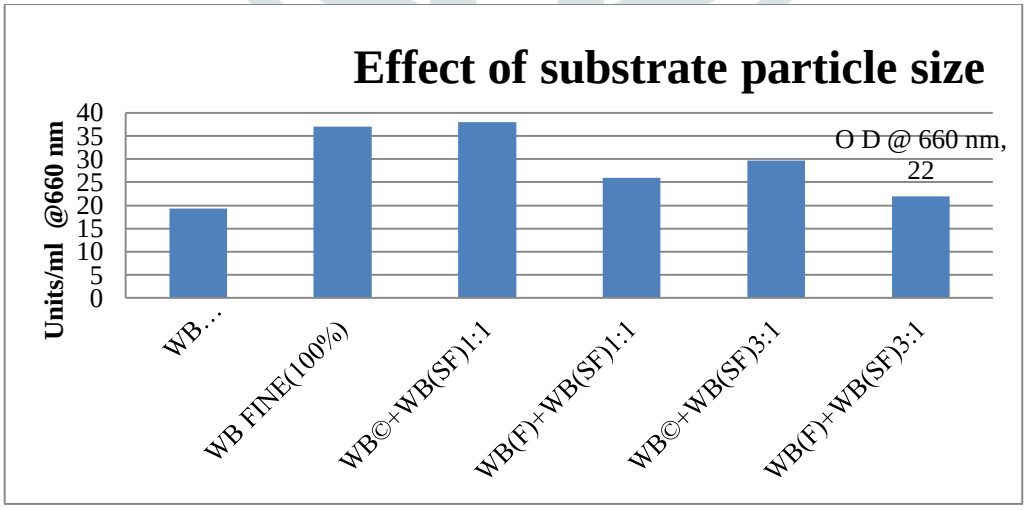

Fig no:* 8: Effect of substrate particle size on protease production by *Aspergillus niger

7) **Effect of substrate moisture content:**

Among the several factors that are important for microbial growth and enzyme production using a particular substrate, particle size and moisture level/water activity are the most critical. The effect of the moisture content on solid substrate fermentation is one of the important factor, which has relative effect on the production of enzymes, particularly during the up scaling process, relative moisture content should be maintained in order to obtain the good yields, mainly when the open tray solid substrate fermentation conditions were carrying on.

Below graph shows the importance of maintaining the proper moisture levels during the solid substrate fermentation, SSF with 150% of moisture content has showed the good results when compared with the SSF with 100 % moisture content hence sufficient moisture content should be maintained to obtain the good yield.

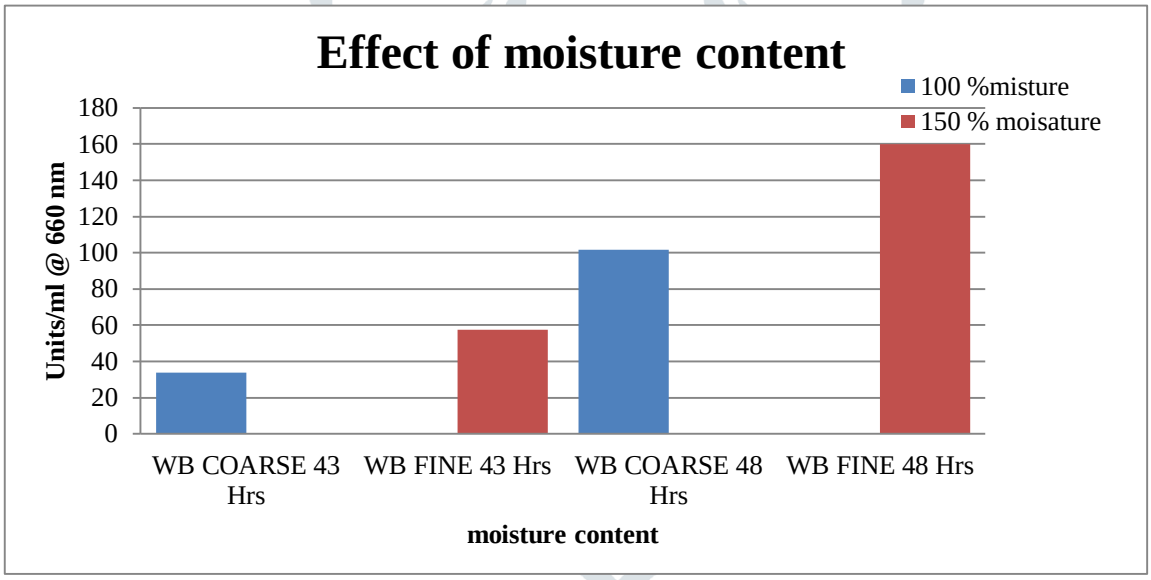

Fig no:* 9: Effect of moisture contents on protease production by *Aspergillus niger

4. CONCLUSIONS

Based on the results of this work, the following conclusions can be drawn:

In this study an industrial strain of *Aspergillus niger* is used for protease production. This strain of *A.niger* is maintained by following methods such as sub culturing and glycerol stocks. Protease production is through solid state fermentation, growing *A.niger* on agro-industrial waste such as wheat

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5. References:

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