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Protease production by *Aspergillus flavus* under solid state fermentation using cotton seeds and sugarcane bagasse

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Abstract
The production of protease by *Aspergillus flavus* under optimized conditions has been studied under solid state fermentation. Cotton seeds and sugarcane bagasse is used as substrates. The best substrate for maximum protease production was found to be sugarcane bagasse and a high protease activity was observed on the 144th hr of growth. A pH value of 4.0 with temperature 40 ^{0}C, inoculums size of 3% of solid substrate and a moisture content of 60% were found to be optimal for maximum protease production. The study proved that locally isolated fungal species is able to produce a very high level of protease under SSF using inexpensive agro-residual substrates, which can be useful in industries.

Keywords: *Aspergillus* sp., protease, solid state fermentation

Introduction
Proteases (peptidases or proteolytic enzymes) constitute a large group of enzymes those catalyses the hydrolysis of peptide bonds in other proteins. Proteases occur in animals, plants and microorganism, and have critical role in many physiological and pathological processes such as protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumor growth and metastasis, activation of zymogens, release of hormones and pharmacologically active peptides from precursor proteins, and transport of secretary proteins across membranes [1].
Microbial proteases are the leaders of the industrial enzyme market worldwide and account for approximately 60% of the total enzyme sale in the world [2]. Among the world sale of industrial enzymes, 75% of these are hydrolytic enzymes, of which two-thirds are proteolytic enzymes [2].
Filamentous fungi are used in many industrial processes for the production of enzymes and metabolites. Among the many advantages offered by the production of enzymes by fungi are low material costs coupled with high productivity, faster production, and the ease with which the enzymes can be modified. Further, the enzymes, being normally extracellular, are easily recoverable from the media [3]. Proteases productions of fungal origin have an advantage over bacterial protease as mycelium can be easily removed by filtration. Besides, the use of fungi as enzyme producer is safer than the use of bacteria, since they are normally recognized as GRAS (generally regarded as safe) [4].
In present study, for protease production SSF (Solid State Fermentation) technique has been used because of its advantages like uses raw materials as substrates, utilization of less energy and space, low production cost, downstream processing, stability of the product due to less dilution in the medium, and manufactures with higher productivity [5, 6].
In recent years there have been many attempts to produce different type of protease through SSF using several different types of substrates by a great number of fungal strains. In present study *Aspergillus flavus* has been used to produce protease enzyme by utilizing cotton seeds and sugarcane bagasse which are common agro-residues produced in large quantity in India.

Materials and Method
Present *in-vitro* study has been conducted in biotechnology department of SV University, Gajraula, UP. The details of material required and method adopted during the investigation are below under appropriate heads.

1. Collection of Materials: Cotton seeds, and sugarcane bagasse were collected from crop fields of University campus. The collected samples was ground in dry blender until the size of the particles reached the desired size and stored at ambient condition for further use as enzyme substrate.

2. Fungal Isolation: Fungal strains of *Aspergillus flavus* was isolated from soil of sugarcane field and cotton crop field respectively. All the traces of plants and weed seeds and grasses were removed from the soil by sieving it and will be used in serial dilution technique [7]. The isolation of fungal species was done on Potato Dextrose Agar Medium.

3. Protease Production by Solid State Fermentation: Ten grams of each substrate i.e. cotton seeds and sugarcane bagasse was taken in a 250 ml Erlenmeyer flask separately. A salt solution of Ammonium chloride-0.5g; Sodium nitrate-0.5g; Potassium dihydrogen orthophosphate-0.2g; Magnesium sulphate-0.2mg; Sodium chloride-0.1g was prepared in 100ml distilled water. Each substrate was moistened with 15 ml of this salt solution. All the flasks has been plugged tightly with cotton wool, and sterilized at 121.5 °C for 15 min. After cooling, each flask was inoculated with 1 ml of fungal spore suspension of *Aspergillus flavus* (100 spores/ml) and incubated at 37 °C for 120 hr [8]. Each experiment was done in triplicates for more accuracy and statistical calculations.

4. Extraction of Protease: A solution of Tween water was prepared by adding Tween-80 (0.1%) to the 100 ml of distilled water. Fifty milliliter, of this Tween water was mixed to the fermented substrate. This substrate was homogenized on a rotary shaker at 120 rpm for 12 h. This homogenized substrate was filtered with help of 4-fold muslin cloth. The suspended solids were removed by centrifuging the homogenate at 8000 x g at 4°C for 15 min. and supernatant was collected in sterilized containers. This supernatant was used for characterization and purification [8].

Optimization of process parameters in Protease Production

(A) Effect of Initial pH of the medium: The effect of initial pH on protease production was studied by changing the initial growth medium pH from 2,4,6 and 8 and 10 before sterilization at 121 °C for 15 min. by addition of hydrochloric acid (0.1 N) and 0.1 N sodium hydroxide to achieve acidity and alkalinity respectively.

(B) Effect of Incubation Temperature: The effect of temperature on activity of protease was studied by taking various temperatures ranges like 30, 40, 50, 60, 70, and 80°C. The optimization media was inoculated with the test samples at different temperatures and the protease assay was done after 24 h.

(C) Effect of Incubation Time: The effect of incubation period on protease was determined by incubating production medium for different incubation periods *viz*. 24, 48, 72, 96 and 120 h.

(D) Effect of Initial Moisture content: The effect of varying moisture content on the production of protease at basal medium conditions including initial moisture content of 50% and 10% inoculum level was studied. The further fermentation will be done under various initial moisture contents (50, 60, 70, 80 &90%) adjusted with distilled water.

(E) Effect of inoculums size: Size of inoculum is a major biological factor in the production of the enzyme. The selected fermentation medium of present study, has been inoculated with various percentage of the fungal strain 1%, 2%,3%,4%and 5%.

Result and Discussion

Protease production by solid state fermentation (SSF) was assessed with both cotton seeds and sugarcane bagasse as substrate for *Aspergillus flavus*. It has been reported that enzyme production is strongly influenced by different culture conditions (physical factors) such as pH, temperature, moisture content, and incubation period and inoculums quantity. Protease production by microbial stains strongly depends on the extracellular pH because pH strongly influences many enzyme process and transport of various components across the cell membrane which in turn supports the cell growth and product production [9]. The results showed in figure 1 illustrates that significant optimum enzyme yield was obtained at pH- 4.0 in both the substrates i.e. cotton seeds and sugarcane bagasse. The comparative graph peaks also indicate that enzyme production level is low in cotton seeds than sugarcane bagasse as a substrate. The results are also in support to the study of Muthulakshmi *et al.* 2011 [10] in which she stated that *Aspergillus flavus* shows its maximum yield at pH 4 while using wheat bran as a substrate.

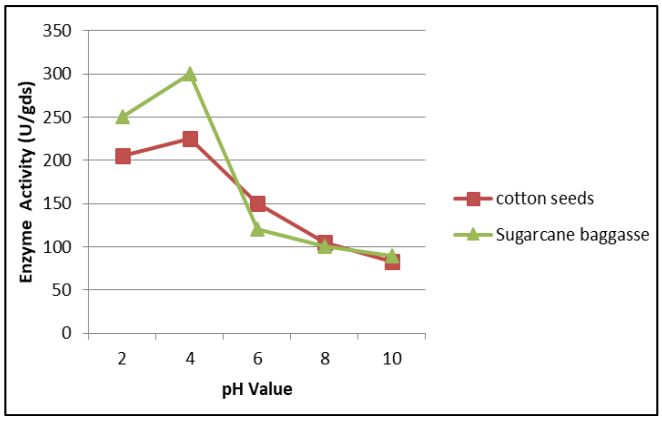

Fig 1: Effect of pH on protease produced by *A. flavus* under SSF using Cotton seeds and Sugarcane bagasse as substrate.

Temperature is considered as most important factor which influences the fungal growth. Figure 2 indicates that optimization study carried out from 20 ^{0}C to 80 ^{0}C and there was an increase in enzymatic production when incubation temperature was increased from 20 ^{0}C -40 ^{0}C. Sugarcane bagasse provides optimum production at 40 ^{0}C but it is increased while increasing the temperature further. In case of cotton seeds intensity of temperature does not affect the yield so much at 30, 40 and 50^{0}C but above 50 ^{0}C the enzyme yield declined rapidly. This declined enzyme production with high temperature is because fungal proteases are usually thermo labile and show reduced activities at high temperature [11]. High temperature is found to have some adverse effect on metabolic activities of microorganism [12] and inhibit fungal growth. The enzyme is denatured by losing its catalytic properties at high temperature due to stretching and breaking of weak hydrogen bonds with an enzyme structure [13]. Results of the present study were found to be very close to the observation of Paranthaman, 2009 [9] where the *Aspergillus niger* produced maximum yield of protease production at 35 ^{0}C.

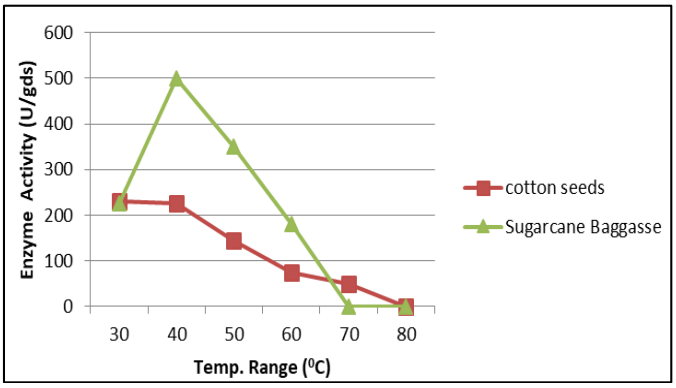

Fig 2: Effect of temperature on protease produced by *A. flavus* under SSF using Cotton seeds and Sugarcane bagasse as substrate

In SSF water must be available in the fermentation medium for microbial growth and biochemical activity. Indeed water content influences the physical state of the substrate, nutrient availability, diffusion of nutrients and Oxygen -carbon dioxide exchange in a complex way [14]. In the present study the optimum yield was obtained with 60 to 70% initial moisture content which decreased with increasing water content as well as in less amount of water (figure 3). Similar result intensity was also observed by Haque *et al.* 2016 [15] where 65% moisture content found to be perfect for maximum enzyme yield. These observations are in support to the explanation that low moisture level is associated with an early sporulation and a low enzyme yield. This may be explained by the non availability of nutrients. High moisture level decrease particles porosity and stickiness of substrate. This results in agglomeration and reduction of gas volume and gaseous diffusion inducing as a consequence of low oxygen transfer [16, 17].

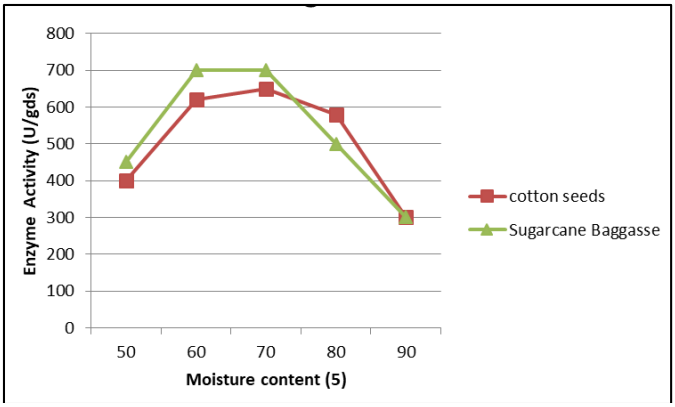

Fig 3: Effect of Moisture content on protease produced by *A. flavus* under SSF using Cotton seeds and Sugarcane bagasse as substrate.

The incubation period is directly related to production of enzymes and other metabolites to a certain extent. After that the enzyme production and the growth of the microorganism decreases; this can be attributed to the reduced availability of nutrients and the production of toxic metabolites [18]. The results presented in figure 4 indicate that increased production of protease increased with the passage of time. The highest enzyme production was observed on 7th day of incubation. Both the substrates showed similar results for optimization of incubation period as the yield increased at its maximum after 144 hours of incubation. However the yield of enzyme was more in sugarcane bagasse then cotton seeds but their intensity of production increase remain same with increase of time. These results were analyzed to be similar by Muthulakshmi *et al*. 2011 [10] and Malathi *et al.* 1991 [19] who reported that high protease production occurs during 7th day of incubation by *Aspergillus* species i.e. *Aspergillus flavus* and Aspergillus niger respectively.

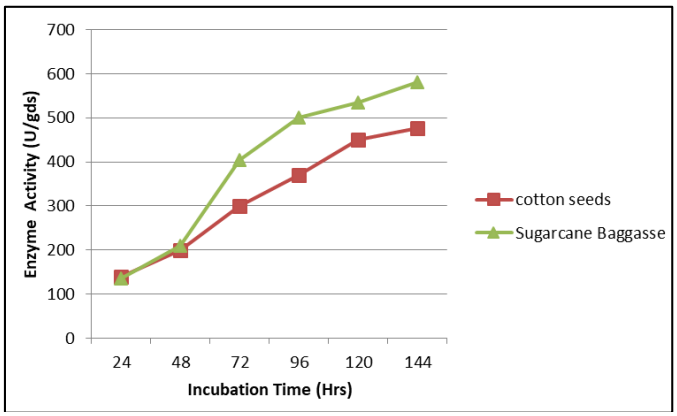

Fig 4: Effect of Incubation time on protease produced by *A. flavus* under SSF using Cotton seeds and Sugarcane bagasse as substrate.

Inoculums' amount is an important biological factor which determines biomass production in fermentation [20, 21]. Maximum yield of enzyme production should be result of a balance between the proliferating biomass and available nutrients [22]. Figure 5 illustrates that maximum protease production was obtained with 3% inoculums size in cotton seeds and sugarcane bagasse. In present study the production of protease by *Aspergillus flavus* increased with an increase in inoculum size up to a level of 3% after which the yield decreases. This yield reduction is because of less nutrient availability. When the amount of mycelium increased, it rapidly consumed majority of substrate for growth and enzyme synthesis decreased [23, 24]. Such type of results were also obtained by Paranthaman *et al*, 2009 [9] in which further increase in inoculums volume resulted to decrease in protease production.

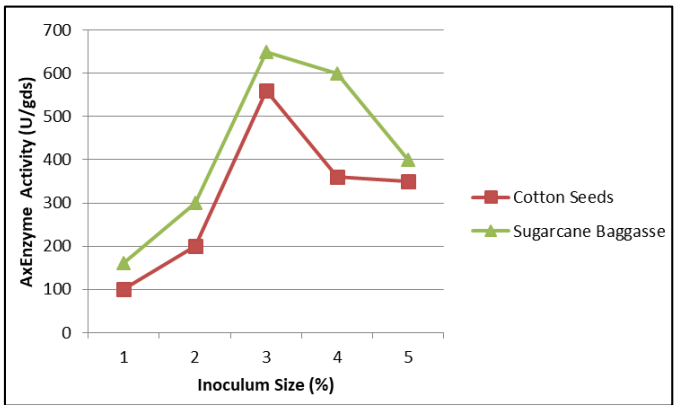

Fig 5: Effect of Inoculums size on protease produced by *A. flavus* under SSF using Cotton seeds and Sugarcane bagasse as substrate.

Present study characterized from a locally isolated fungus *Aspergillus flavus*. The study also suggest that cotton seeds and sugarcane bagasse has a good potential for protease production using *Aspergillus flavus* under solid state fermentation. However the study also provides a comparative account of both the substrates. This preliminary comparison promotes the use of agricultural residues as a low-cost medium eco-friendly substrate for enzyme technology.

Conclusion

The results of the study conclude that composition of the medium and optimization culture condition is a major factor to regulate the extracellular enzyme synthesis in SSF. The growth and yield of enzyme is based on selection of suitable substrate, fermentation medium, optimize pH and temperature, appropriate moisture, incubation time and suitable quantity of inoculums. This research work provides promising results for protease production under optimized condition by which *Aspergillus flavus* has been identified as great producer of extracellular protease under SSF.

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