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Screening of Protease Produced by *Aspergillus Flavus* Under Solid State Fermentation Using Rice Bran, Cotton Seeds and Sugarcane Bagasse

Apoorva Gaur[1], Garima Bartariya[2]

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Abstract

The production and estimation of protease by *Aspergillus flavus* has been done by using cotton seeds, rice bran and sugarcane bagasse as substrates. OD was recorded at 660 nm. Protease activity and specific activity was calculated daily till 6th day of incubation. The best substrate for maximum protease production was found in sugarcane bagasse and high protease estimation has been observed between 4-7 days of incubation. 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; Protein assay.

Introduction

Protease is an enzyme that breaks the peptide bonds of proteins.[1] Protease breaks to produce amino acids and other smaller peptides. Protease is the single class of enzymes which occupy a pivotal position with respect to their application in both physiological and commercial fields.[2,3] Extracellular proteases catalyze hydrolysis of polypeptides into free amino acids or smaller polypeptide chains in the outer cell environment and allow the cell to take up and utilize product of hydrolysis.[4] Microbial proteases are directly excreted into production medium due to their extracellular nature, thus simplifying purification of enzyme as compared to enzymes extracted from animals and plants.[5] Considering the importance of proteases it is necessary to screen and isolate novel protease producing microbes from different sources. It is ideal that proteases isolated from microbes should maintain higher activities over a broad range of temperature, pH etc. to be used in various industrial applications.[6]

Author's Affiliation: [1]Research scholar, [2]Assistant Professor, Department of Biotechnology, Shri Venkateshwara University, Gajraula, Uttar Pradesh 244236, India.

Corresponding Author: Garima Bartariya, Assistant Professor, Department of Biotechnology, Shri Venkateshwara University, Gajraula, Uttar Pradesh 244236, India.

E-mail: dr.garimabartariya@gmail.com

Solid state fermentation (SSF) using agro-industrial residues promotes the production of enzymes at a lower cost and in eco friendly way. SSF uses agro-industrial wastes, like seeds, peels, husks, bark, and bran to produce valuable bioactive molecules. Filamentous fungi of industrial interest include several *species* of Aspergillus sp.,[7] as they are capable of substrate adaptation and produce several metabolites with high biological activities. The *genus* Aspergillus is also considered non-toxic, recognized as a safe microorganism by the Food and Drug Administration (FDA), denominated Generally Recognized as Safe (GRAS), and used for human and animal nutrition.[8,9,10]

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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

Rice Bran, cotton seeds, and sugarcane bagasse were collected from crop fields of University campus. The collected samples were 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 rice field, 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 - used in serial dilution technique.[11] 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. rice bran, 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 (12). 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 × g at 4°C for 15 min. and supernatant was collected in sterilized containers. This supernatant was used for characterization and purification.[12]

5. Estimation of protease activity

Protease activity in the crude enzyme extract produced by using different substrates i.e. rice bran (RB), sugarcane bagasse (SB) and cotton Seeds (CS) was determined by following the methodology of Carrie Cupp Enyard, 2008.[13] For reaction, a mixture with 5 mL of 0.65% casein solution in 50 mM potassium phosphate buffer, and 1 mL of crude enzyme extract was prepared at pH 7.5 and incubated at 37 °C for 30 minutes. After that 5 mL of Trichloroacetic acid solution (110 mM) was added to regulate the reaction. On settling undisturbed for 15 minutes the mixture was filtered using Whatmann's No 1 filter paper. Then 2 mL of filtrate was mixed with 5 mL of sodium carbonate solution (500 mM) and 1 mL of 2 fold diluted Follin Ciocalteus phenol reagent. The obtained mixture was kept in dark at room temperature for 30 minutes. Appearance of blue color was observed in the solution after measuring time. The optical density (OD) was measured at 660 nm against a reagent blank using tyrosine standard. The concentration of liberated tyrosine (OD) in solution was estimated by comparing with standard curve.

Standard curve of tyrosine was prepared by using the concentration range of 27.5, 55, 110, 220 and 275 µM (Figure 1). One protease unit was defined as the amount of enzyme required to releases 1 µM of tyrosine per minute per mL at 37 °C, pH 7.5.[14] All the experiments were carried out in triplicates for more accuracy. The enzyme activity (U/mL) was calculated by following formula:

$$\text{Enzyme activity (Units/mL)} = \frac{\text{µmole tyrosine equivalent releases} \times 11 \text{ (Total volume of assay)}}{\text{Volume of enzyme taken (1 mL)} \times \text{Incubation time (30)} \times (2)}$$

11 = Total volume of assay (in milliliters).

1 = Volume of enzyme used (in milliliters).

2 = Volume of sample taken in cuvette for absorbance.

Specific activity is the activity of an enzyme per milligram of total protein (expressed in µmol/min/mg). The specific enzyme activity (U/mg) was calculated by following formula:

$$\text{Specific enzyme activity (U/mg)} = \frac{\text{Enzyme activity (U/mL)}}{\text{Total protein content (mg/mL)}}$$

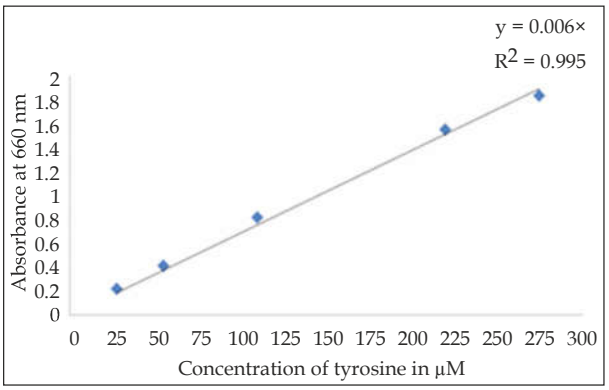

Figure 1: Standard curve of Tyrosine.

6. Protein assay

Total protein estimation in the crude enzyme solution was done by following Folin- Lowry method. The observation was recorded by considering Bovine Serum Albumin (BSA) as standard at 750nm.[15] Standard curve of BSA was prepared by taking BSA in following concentration range: 40, 80, 120,160, and 200 µg/mL. (Fig. 2)

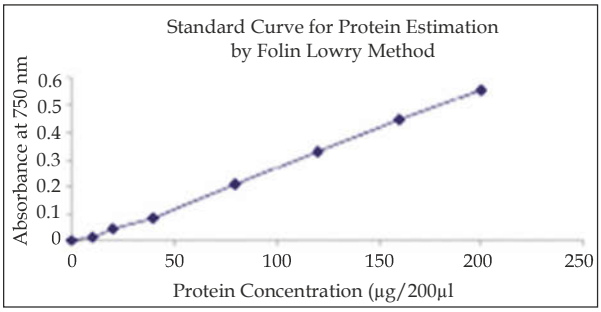

Figure 2: Standard curve of BSA.

Result and Discussion

Protein activity in the culture filtrate was tested using casein as protein substrate. Table-1 showed protease production and specific activity of *Aspergillus flavus* from three different substrates i.e. rice bran, cotton seeds and sugarcane bagasse. Maximum protease activity (72.16 ± 5.25) was obtained from sugarcane bagasse after 6 days of incubation whereas highest specific activity i.e. 42.45 ± 1.85 was also present in sugarcane bagasse. When compared to all the three selected substrates, rice bran was found least productive for protease, however its production rate (42.04 ± 1.66 cannot supposed to be very low but other two substrates i.e. cotton seeds and sugarcane bagasse were slightly higher than this. A comparison of the data indicated nearly similar pattern of protease activity in all the selected substrates. Similar intensity of the results for different soil samples were observed by Sharma et al., 2015. Production of alkaline protease by *Asprgillus fumigatus* has been reported earlier by Monad et al., 1990[16] and Hossain et al., 2016.[17]

The results revealed that all selected substrates are best agro-industrial waste material that supports maximum protease production by *Aspergillus flavus*. Besides a good inducer for proteolytic enzyme production they are also agro-industrial residues from common crops which are of low cost and always available. From the present study it is also concluded that sugarcane bagasse is an excellent substrate among them in SSF as it is a biennial crop and always available. It also provide adequate amount of nutrients to support fungal

Table 1: Protease production and specific activity from 3 different substrate during 6 days of incubation.

Incubation Days	O.D. at 660nm			Protease activity (U/mL)			Specific activity (U/mg)		
	RB	SB	CS	RB	SB	CS	RB	SB	CS
Day 1	0.451	0.523	0.477	7.31±5.73	20.91±1.82	19.04±2.66	2.99±1.92	12.05±1.39	10.26±2.07
Day 2	0.726	0.801	0.934	15.87±4.11	26.46±1.35	22.55±1.02	4.17±1.02	16.83±1.88	15.50±1.88
Day 3	0.933	0.992	1.002	42.04±1.66	34.68±0.79	29.58±0.44	18.22±2.01	20.77±0.64	18.09±4.13
Day 4	1.005	1.258	1.251	30.65±1.22	40.79±1.01	36.83±0.24	12.19±2.78	24.27±0.99	24.64±0.78
Day 5	1.055	1.521	1.456	32.28±0.64	52.58±3.11	40.33±1.21	17.39±2.05	36.47±1.43	38.45±2.74
Day 6	1.258	1.954	1.652	36.77±4.62	72.16±5.25	56.13±0.14	10.68±1.23	42.45±1.85	22.86±1.61

growth form maximum protease production. Figure 1 shows protease production in SSF through 6 days of incubation. It can be seen that most substrates support fungal growth and protease secretion between 4-6 days of incubation.

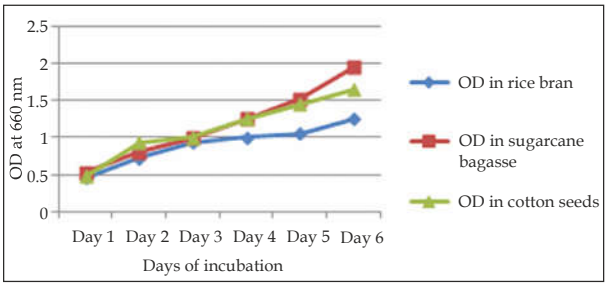

Figure 1: Comparison in Optical Density at different days of incubation in three substrates during protease production

Cost and efficiency are major characteristics of enzymes for industrial applications. Hence ,it is desirable for the material used in the fermentation medium to be of low cost, available in large amount and continually renewable , which is exactly the case of Agro industrial residue, in addition to making microbial growth possible.[18]

Conclusion

Production of protease by microbial strains exhibits a characteristic relationship with selection of substrate in optimal condition. Considering many industrial applications of protease, this study has provided promising results with protease production from different proteinaceous substrates. *Aspergillus flavus* has been identified as great producer of extracellular protease in fermentation techniques. Microbial production of protease has got a great attention in recent years because of their biotechnological potential in various industries.

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