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PRODUCTION OF CELLULASE BY SOLID STATE FERMENTATION

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ABSTRACT
The present work deals with optimization of cultural conditions for the production of cellulases by Aspergillus niger MTCC 2196 using agricultural byproduct. A.niger gave maximum production of cellulases by solid state fermentation using green gram husk than black gram husk. The cultural conditions such as Spore count (1×10^8 spores/ml), incubation period (8 days), pH (5.0), temperature (28±2 °C), particle size (50 Mesh), thickness (1mm), and moisture content (89.35%) were studied.

Key words: *Cellulases, green gram husk, Aspergillus niger MTCC 2196, solid state fermentation etc.*

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INTRODUCTION

Cellulases are industrially important enzymes that are sold in large volumes for use in different industrial applications, like Starch processing, animal feed production, grain alcohol fermentation, malting and brewing of fruit and vegetable juices etc. (Ogel et. al., 2001 ; Maryam *et. al*., 2007).

Submerged fermentation (SmF) is used for industrial production of cellulase. The cost of production and low yield of these enzymes are the major problems for industrial applications (Kang *et. al*., 2004). Solid state fermentation (SSF) which is economical due to its lower capital investment and lower operating expenses has been reported as an

alternative process to produce cellulases (Panagiotou et. al., 2003 ; Yang et al., 2004).

Solid state fermentation is the cultivation of microorganisms on moist solid raw materials, in the absence of free aqueous phase, that is at average water activities (defined as the relative humidity of the gaseous phase in equilibrium with the moist solid) significantly below 1 (Pandey *et .al*., 2003). This is an alternative to the cultivation of microorganisms in liquid nutrient broths (SmF).

The present work is concerned with the exploitation of agricultural byproducts for the production of industrially important enzyme, cellulases by the solid state fermentation using *A.niger*.

MATERIALS AND METHODS

Chemicals

All chemicals and medium constituents used for the present study were procured from M/s Hi-media, Bombay (India).

Microorganism

Aspergillus niger MTCC 2196 was obtained from Microbial Type Culture Collection (MTCC) of IMTECH, Chandigarh. It was maintained on PDA slants at 4^0C and subcultured once ina month.

Inoculum Preparation

Spores were harvested from a 9 day old PDA slant in 5 ml of sterile distilled water. Appropriate dilutions were made and then 2 ml of spore suspension was added to the pretreated substrate based medium.

Spore count

Various dilutions of spore suspensions were prepared using sterile distilled water. The dilutions were made for the original 5 ml of spore suspension prepared from a 9 day old fungal slant. These dilutions were 1:1, 1:20, 1:50 and 1:100. 2 ml each of the diluted spore suspensions were separately used to inoculate moist pretreated green gram husk. The moist pretreated green gram husk was previously sterilized by autoclaving (Lab Tech LAC-5060S) the petriplates at 121^0C and 15 psi for 20 minutes. After inoculation the petriplates were incubated (Lab Tech LSI-3016A2) at 28± 2^0C in an incubator for 8 days. The mouldy substrate was then harvested and the biomass in terms of protein content was estimated.

Incubation period:

Effect of incubation period was determined by incubating fermentation medium for one to ten days in BOD incubator (IRI-022). The samples were collected at an interval of 24 hrs. The mouldy substrate was then harvested and the biomass in terms of protein content was estimated.

Effect of pH:

The pH was varied from acidic to neutral to alkaline range. Pretreated green gram husk (PGGH) was moistened with water in all the Petri plates. The water used for moistening was also used for the pH adjustment. pH of water was varied from 1.0, 3.0, 5.0, 7.0 and 9.0 and then equal quantities of each were used for moistening the pretreated green gram husk in different petriplates. These petriplates were then autoclaved (Lab Tech LAC-5060S) at 121^0C and 15 psi for 20 minutes and were inoculated with 2 ml of spore suspension under sterile conditions. These petriplates were then incubated at 28±2^0C in an incubator (Lab Tech LSI-3016A2) for 8 days. The mouldy substrate was then harvested and the biomass in terms of protein content was estimated.

Temperature effect:

The temperature during incubation period was varied at 22±2^0C, 28±2^0C, 32±2^0C, and 60^0C for different petriplates containing moist pretreated green gram husk. The pH was adjusted at 5.0 with water. The petriplates were then autoclaved (Lab Tech LAC-5060S) at 121^0C and 15 psi for 20 minutes and inoculated with 2 ml of spore suspension under sterile conditions. These petriplates were then incubated (Lab Tech LSI-3016A2) at different temperatures for 8 days. The mouldy substrate was harvested and the biomass in terms of protein content was estimated.

Particle size:

The pretreated green gram husk particle size was varied but the pH was kept in 5.0 in all petriplates. 20, 30, 50 and 80 mesh sizes were used to setup substrate in different petriplates. The petriplates were autoclaved (Lab Tech LAC-5060S) at 121^0C and 15 psi for 20 minutes. These were then inoculated with 2 ml of spore suspension under sterile conditions. These petriplates were then inoculated at 28 ± 2^0C in an incubator (Lab Tech LSI-3016A2) for 8 days. The mouldy substrate was then harvested for analysis.

Thickness:

Pretreated green gram husk was spread evenly in a glass petriplate such that its thickness was measured using a scale. The moisture was provided to the substrate such that there was no free liquid. The thickness of the substrate was equal at all the regions of the petriplate. All the petriplates had 50 mesh size PGGH at a pH of 5.0. The petriplates with substrate at various thicknesses were autoclaved (Lab Tech LAC-5060S) at 121^0C and 15 psi for 20 minutes. These were then inoculated with 2 ml of spore suspension under sterile conditions and incubated at 28 ± 2^0C in an incubator (Lab Tech LSI-3016A2) for 8 days. The mouldy substrate was harvested and the biomass in terms of protein content was estimated.

Moisture:

Pretreated green gram husk was spread evenly at 1 mm thickness in petriplates. The moisture was varied in these petriplates. The pretreated green gram husk in each of the petriplates was made wet without any free liquid. The weight of wet pretreated green gram husk was noted as initial weight. All these weights were considered equivalent to 100% moisture level for the wet pretreated green gram husk in that particular petriplate. These petriplates were kept in an oven at 40^0C for varying time intervals i.e., 5, 10, 15, 20, 25 minutes and so on. The difference in the final and initial weights of the wet PGGH was used to calculate moisture level in these plates. The procedure was performed in triplicates to arrive at a reliable value for a particular moisture percentage.

After observing the trend of change in the moisture level 1 mm thick layers of pretreated green gram husk at a pH of 5.0 and 50 mesh particle size were prepared and autoclaved (Lab Tech LAC-5060S) at 121^0C and 15 psi for 20 minutes. These were inoculated with 2 ml of spore suspension under sterile conditions and incubated at 28 ± 2^0C in an incubator (Lab Tech LSI-3016A2) for 8 days. The mouldy substrate was then harvested and the biomass in terms of protein content was estimated in order to obtain the ideal moisture level for cultivation of *Aspergillus niger* MTCC 2196 to obtain high biomass yields.

Enzyme assay:

The cellulase estimation was done by the method of Mendel's and Weber (1969).50 mg of Whatman No.1 filter paper strips were incubated with 1 ml acetate buffer (pH 4.8) and 0.5 ml of undiluted sample of the extracted enzyme at 50^0C for 1 hour. The reducing sugars released were estimated by the dinitrosalicylic acid (DNSA) reagent (Miller, 1959). The cellulase enzyme activity was expressed in terms of milligrams of reducing sugars released per milliliter of the enzyme extract.

Protein estimation:

The protein was measured in the culture supernatant, and estimated by Modified Lowry's method (1951).

RESULTS AND DISCUSSION

Spore count

Various dilutions of spore suspension were made to find most suitable spore count and standardize the inoculums size. A high enzyme yield was obtained when1×10^8 spores/ml (counted on haemocytometer) by using Motic Digital Zoom Microscope were used to inoculate the SSF medium (Table, 1 Plate,1).

Spore count was necessary for ideal cultivation and higher enzyme yield from biomass. If the spore number was low i.e, dilute suspension, it led to slow growth and longer time to utilize the

substrate. On the other hand, if the spore count was very high, i.e, the suspension was concentrated, then the media surface becomes too crowded and there was severe competition among the individual hyphae which caused and adverse on growth and final enzyme yield.

Dilution of spore suspension	Enzyme yield on 8th day of growth (mg/g PGGH)
1:1	7.1
1:10	13.2
1:20	36.4
1:50	12.2
1:100	2.3

Table 1: Effect of various dilutions of spore suspension used as inoculum on growth yield

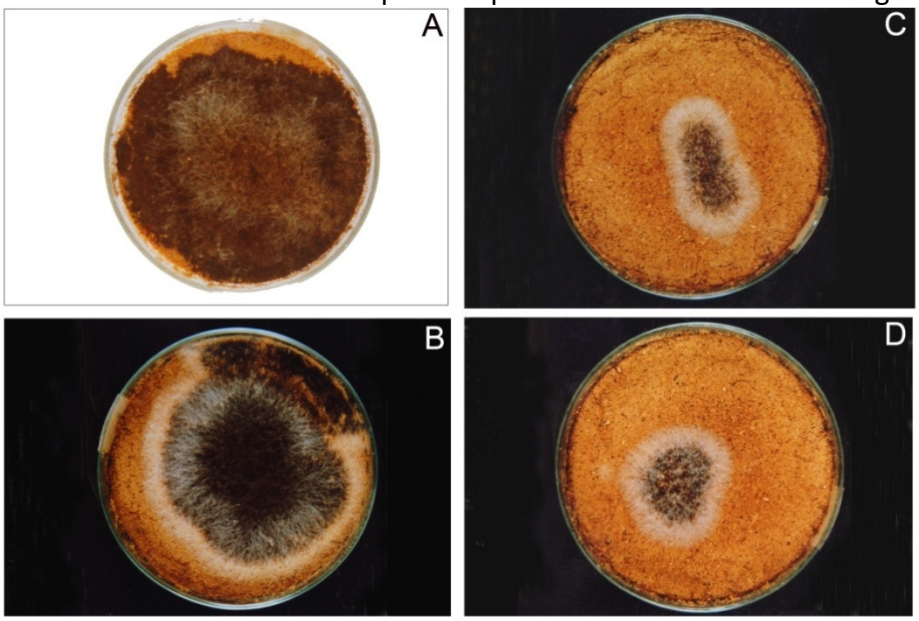

Plate 1: Effect of varied inoculums size on the growth of *A.niger* various dilutions as A. 1:1; B. 1:20; C.1:50; D. 1:100

Incubation Period

A. niger showed a growth period of approximately around 10 days. The initial stage was the vegetative phase. The multiplication of hyphae took place and the biomass as well as enzyme increased during this stage. Also the food materials got accumulated in the hyphae. During the second phase of the life cycle the reproduction occurred. Here the sporulation took place under the adverse conditions. The spores were produced to tide over the unfavourable environmental conditions like reduced carbon source, nutrients in the medium etc. Spores contained the stored food inside a hard cell wall that could be used by the future generation immediately on germination. So, on harvest the enzyme yield was drastically reduced

during the reproductive phase of life cycle. This stage was reached in *A. niger* on the 9th day when grown on the PGGH. In this organism conidium instead of spores was produced without conidiomata (Singleton and Sainsbury, 1988). Hence the harvest for the enzyme was made on the 8th day in all the experiments to avoid conidia (Fig 1).

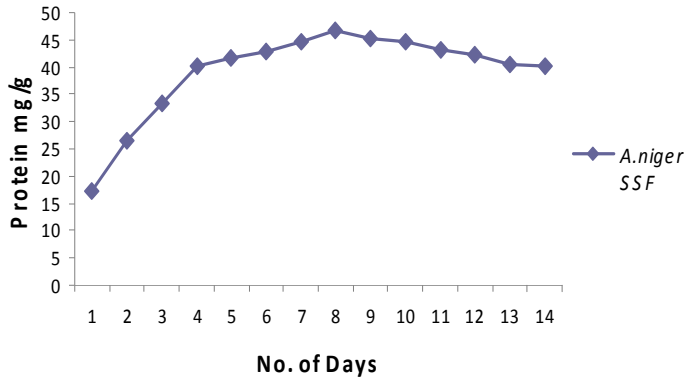

Fig 1 Growth Curve *of Aspergillus niger* on pretreated green gram husk by SSF

pH

Initial PH for the medium was varied and its effect was studied in terms of enzyme yield by *A. niger* when grown on GGH by SSF (Fig 2,). pH 5.0 gave highest yield in terms of enzyme yield for both PBGH and PGGH. It was 6.246 and 34.124 ml/gm of PBGH and PGGH respectively. General trend in both the cases showed that PH in extremely acidic conditions showed a lower growth. As acidic nature approached the neutral pH enzyme yield improved to maximum and then again declined. Among all the conditions the most suitable was slight acidic nature of the medium. It was also observed that high sugar and low pH conditions, which are adverse to bacterial growth, but were ideal for *A.niger*. The advantage of this pH was also that it was partially selective in avoiding the bacterial contamination.

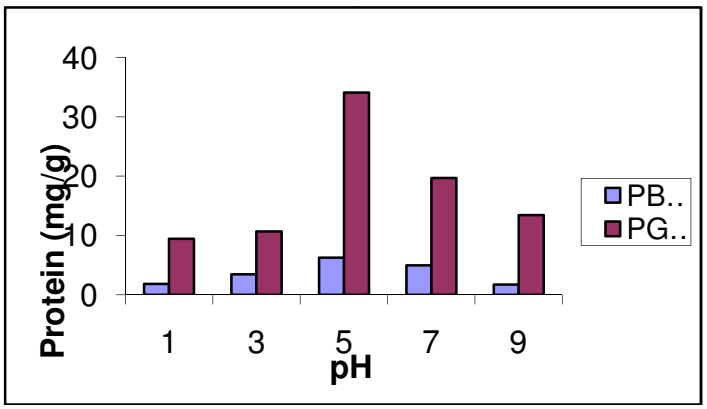

Fig 2 Effect of variation of initial pH of SSF medium on the protein yield by *A. niger*

Temperature

Among the various temperatures tested 28±2^{0}C gave highest enzyme yield (Fig 3). Extreme temperatures affected the growth statistics. At low temperature i.e., below 25^0 C there was a decline in the growth. Here the cellular activities stopped due to low temperature and no growth was observed. At high temperature like 60^{0}C the growth was almost stopped. It was because all the cellular activities stopped due to extremely dry and heated conditions of the medium.

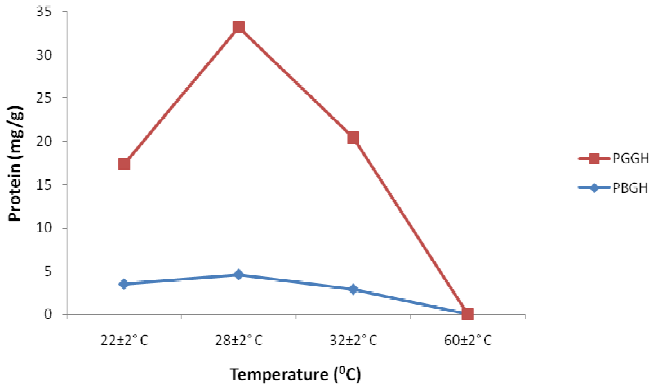

Fig 3 Effect of temperature on protein yield by *A.niger*

Particle size

A particle size of 50 mesh (0.3 mm) was found most suitable among 80, 50, 30 and 20 mesh sizes. The growth yields declined when the particle size was increased or decreased beyond 0.3 mm (Table 2). This was due to reduced growth as cellulose

utilization efficiencies decreased and subsequent low cellulase yield reduced the free sugar concentration in the medium causing a further decline in the growth and enzyme yield.

Mesh size	Enzyme yield on 8th day of growth (mg/g PGGH)
80	29.69
50	40.23
30	24.22
20	21.19

Table: 2 Effect of particle size of substrate on growth yield

Thickness

A substrate thickness of 1 mm gave maximum yield of enzyme when compared to that of other thicknesses (Fig 4). At higher thicknesses the yield declined. This was because the porosity on 1 mm thick substrate supported the easy penetration of the fungal mycelium thus promoting its growth as well as biomass yield. Penetration and entangling of hyphae with the substrate helped in the release of more hydrolytic enzymes. Hence more free sugars were released in the vicinity of the growing hyphae to be directly absorbed by the fungi and used for the growth. Also, this thickness was ideal for gaseous exchange. The higher thickness made the substrate non-porous and harder for hyphae to penetrate and grow efficiently. Hence, only superficial substrate layer was utilized for the growth and most of the lower layers of the substrate remained unused and were wasted.

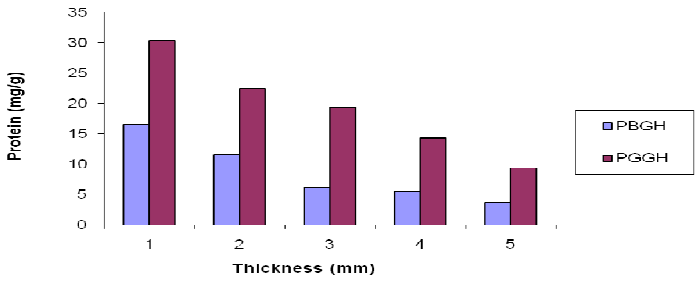

Fig 4 Effect of thickness on the protein yield using *A.niger* by SSF

Moisture

89.35% of moisture content gave the best enzyme yield (Fig 5). The moisture percentage greater and lower than this value caused a decline in the enzyme yield. High moisture content in the medium caused free flowing water in the substrate. This decreased the porosity, lowered O_2 and nutrient diffusion rates and reduced gaseous exchange leading to growth of aerial mycelium. The lower moisture percentage led to a sub-optimal growth as it lowered the degree of substrate swelling and surface area of substrate for attack and utilization by the organism. Hence, only a particular percentage of moisture was required for specific substrate and also for pretreated green gram husk for the ideal and efficient growth of selected strain.

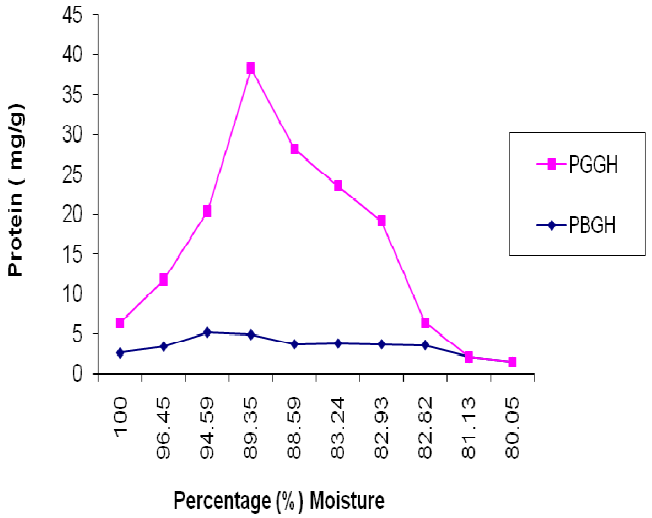

Fig 5 Effect of variation of moisture in SSF medium on protein yield by *A.niger*

CONCLUSION:

The potential of agro wastes especially food wastes have been identified. The selected waste i.e. green gram husk, novel substrate is abundantly available in the country and in particular Andhra Pradesh, was quite feasible for cellulase production. The strain *Aspergillus niger* MTCC 2196 was found to be most suitable for production of cellulase.

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