



## Cellulase Production by Utilizing Agricultural Wastes

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### Abstract

Cellulases are the group of hydrolytic enzymes and they are capable of degrading all types of lignocellulosic materials. Cellulases have wide range of applications. Present work focuses on the factors for improvement of enzymatic hydrolysis of various agricultural wastes by using different fungal species. It was the aim to study the Cellulase enzyme production ability of fungal strains against the lignocellulosic bio-waste like Rice husks, Millet husks, Banana peels, Wheat bran, Coir waste and saw dust. Simultaneously the effect of different environmental factors such as pH (3–9), substrate concentration (1%–6%) and temperature (10<sup>o</sup>C – 50<sup>o</sup>C). The enzyme assay was carried out by two methods CMCase and FPase and the production was analyzed individually by Miller's modified method of Dinitrosalicylic acid (DNSA). In this study the high level of enzyme production was obtained at 30<sup>o</sup>C and 40<sup>o</sup>C by using Rice husks, Banana peels, Millet husks, Wheat bran, saw dust and Coir waste by using varying substrate concentrations. Out of all the six used substrates banana peels gave the higher production of the cellulase enzyme that is 12.4 units / ml after 4 days of incubation.

**Keywords:** Cellulase, agricultural wastes, fungal strains.

### Introduction

Agricultural and industrial wastes are among the causes of environmental pollution. Their conversion into useful products may ameliorate the problems they cause. These wastes which include mainly leaves, straws, cereals, corncobs etc., in many countries, these materials are generally used as animal feeds. A huge amount of these materials are left on farmlands to be decomposed by microorganisms such as Bacteria and fungi.

Cellulose is commonly degraded by an enzyme called cellulase. This is produced by several microorganisms, commonly by bacteria and fungi<sup>1,2</sup>. Fungi are the main cellulase producing microorganisms, though a few bacteria and actinomycetes have also been reported to yield cellulase activity. For complete hydrolysis of cellulose to glucose, cellulase systems must contain endo-1, 4  $\beta$ -glucan (1, 4  $\beta$ -D-glucanohydrolase' EC 3.2.1.4), exo-1, 4  $\beta$ -glucan (1, 4  $\beta$ -D-cellobiohydrolase' EC 3.2.1.9) and  $\beta$ -glucosidase ( $\beta$ -D-glucosylhydrolase' EC 3.2.1.91) or cellobiase. Thus the hydrolysis of cellulose is completed by the synergistic action of endo- and exoglucanases<sup>3</sup>.

Effective conversion of recalcitrant lignocellulose to fermentable sugars requires three sequential steps: i. size reduction, ii. pretreatment or fractionation and iii. enzymatic hydrolysis<sup>4,5</sup>. One of the most important and difficult technological challenge is to overcome the recalcitrance of neutral lignocellulosic materials, which must be enzymatically hydrolyzed to produce fermentable sugars<sup>4,6</sup>.

### Material and Methods

**Selection of Agricultural wastes:** Rice husks, Millet husks, Banana peels, Wheat bran, Coir waste and saw dust were selected for the cellulase production and these wastes collected from the different areas of the Nanded city.

**Selection and Screening of Cellulolytic Fungi:** The six strains of fungal strains such as *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus flavus*, *Penicillium chrysogenum* and *Fusarium moniliforme* were selected for the cellulase production and collected from the department. Collected samples were spot inoculated on separate sterile 1% CMC containing Potato dextrose agar plates. The plates were incubated for a period of 5 days at 30<sup>o</sup>C. The growth of fungal colonies was observed after incubation period. The fungal species grown rapidly on the plate with zone was selected for the further procedure.

**Pre-treatment of substrate:** Raw substrates were sun dried individually to reduce the moisture content to make them more susceptible for crushing. The crushed substrates were then sieved individually to get powdered form. Then substrates were soaked individually in 1% Sodium Hydroxide solution, in ratio 1:10 (substrate: solution) for 2 hours at room temperature. After which they were washed with distilled water to remove the unwanted chemicals. After washing distilled water was added in ratio 1:10 (substrate: distilled water) and autoclaved at 121<sup>o</sup>C for 1 hour. The treated substrates were then filtered and washed with distilled water until the wash water become neutral<sup>7,8</sup>.

**Inoculum preparation:** The selected culture of *Aspergillus niger* were maintained as stock culture on Potato dextrose agar slants. It was grown at 30<sup>o</sup>C for 5 days and then stored at 4<sup>o</sup>C

for regular sub culturing. 50 ml of inoculum was prepared using potato dextrose broth in 250 ml conical flask. The inoculum was kept in shaker (200 rpm) at 30°C for 48 hours before it was used for the fermentation process.

**Fermentation process:** Freshly prepared 5 % inoculums of *Aspergillus niger* was inoculated into 100 ml of optimized culture medium (L-glutamic acid 0.03 gm, NH<sub>4</sub>NO<sub>3</sub> 0.14 gm, KH<sub>2</sub>PO<sub>4</sub> 0.2 gm, CaCl<sub>2</sub> 0.03 gm, MgSO<sub>4</sub> 0.03 gm, Protease peptone 0.75 gm, FeSO<sub>4</sub> 0.50 gm, MnSO<sub>4</sub> 0.16 gm, ZnSO<sub>4</sub> 0.14 gm, Tween 80 20%, Substrate 3 gm, Distilled water 100 ml) having different substrates in separate 250 ml Conical flasks with reference flasks. All flasks were incubated at 30°C for 9 days (0 to 216 hours). The Cellulase activity was measured at regular intervals.

**Effect of incubation period on Cellulase production:** For determination of time course for cellulase production was carried out by measuring cellulase activity at regular intervals of 48 hours up to 216 hours.

**Determination of reducing sugar and Cellulase activity: CMCase Assay:** The Endogluconase, Carboxymethyl cellulase (CMCase) activity was determined according to Wang et al. 1ml of the crude enzyme supernatant was incubated with 1 ml of 1% CMC in 0.1 M Sodium acetate buffer solution of pH 5.0 for 30 min at 63°C. The resulted reducing sugars were determined according to Miller's modified method of DNSA<sup>9</sup>.

**FPase Assay:** Total cellulase ( FPase ) activity was determined as described by Gadgil et al. 1 ml of the crude enzyme supernatant was incubated with 2ml of 0.1 M citrate buffer of pH 4.8 containing 50 mg Whatman No. 1 filter paper. After incubation for 1 hour at 50°C, the resulted reducing sugars were determined according to Miller's modified method of DNSA<sup>10</sup>.

**Miller's modified method of DNSA:** The culture filtrate was collected from the fermentation media by centrifugation. 1 ml of culture filtrate was taken in a test tube, 1ml of 1% cellulose solution and 1 ml of distilled water was added into it. Control tube was made by adding 1ml of 1% cellulose solution and 2 ml of distilled water. Allowed it to react for 30 minutes. Then 3 ml of DNSA reagent was added in each test tube. The contents of test tubes were heated in boiling water bath for 5 minutes. After heating, contents were allowed to cool at room temperature. At the time of cooling, 7 ml of freshly prepared 40% Sodium Potassium Tartarate solution was added. After cooling, the samples were read at 540 nm by using Spectrophotometer. The amount of reducing sugar was determined by using standard graph<sup>11</sup>.

**Determination of Cellulase enzyme assay:** The cellulase enzyme was assayed by measuring the amount of glucose released from the substrates following the secretion of cellulase enzyme by the organism. The determination of glucose liberated from the substrate was done using the Miller's modified method

of DNSA. 1 unit of cellulase activity was defined as the amount of enzyme which releases 10 ug of glucose in the 30 minutes under specified condition<sup>12</sup>.

**Effect of substrate concentration on cellulase production:** Different concentrations of the substrates ranging from 1% to 6% (w/v) were added to the optimized culture medium in separate conical flasks. Inoculum medium of *Aspergillus niger* was inoculated into these fermentation media that is optimized culture medium and incubated at 30°C for 4 days. After incubation period, enzyme activity was determined by FPase assay method.

**Effect of Temperature on cellulase production:** The optimized culture medium was prepared using the individual substrates with the pH varied from 10°C to 60°C. Inoculum medium of *Aspergillus niger* was inoculated in these optimized culture medium and incubated at respective temperatures for 4 days. After incubation period, enzyme activity was determined by FPase assay method.

## Results and Discussion

In the present work we select the different agricultural wastes which are present abundantly in the environment for the production of Cellulase enzyme. Out of selected five fungal species *Aspergillus niger* was observed as a rapidly cellulose degrading fungi because it gave the rapid and higher growth on the 1% CMC containing potato dextrose agar plate.

Incubation period on cellulase production was determined. It was seen that the banana peels gave the higher production that is 12.2 units / ml after 4 days of incubation. After that second highest production was obtained from rice husks and millet husks that are 12.2 unit / ml and 10.6 unit / ml respectively after 4 days. 8.65 unit / ml production was obtained from the Millet husks after 4 days. And the lowest production that is 9.2 unit / ml was obtained from the wheat bran after 4 days and 9.2 unit / ml and 7.3 units /ml was obtained from the coir waste and saw dust after 5 days respectively.

Substrate concentrations of 1 % to 6 % were considered for the production of cellulase. It was observed that the banana peels, rice husks and wheat bran gave the higher production at 3 % substrate concentration. After that second highest production was obtained from Millet husks, coir waste and saw dust at 4 % substrate concentration. And the lowest production was obtained from the maize cobs at 4 % substrate concentration. The decrease in activity beyond maximum substrate concentration that is 5 % may be due to the inhibitors. This is supported by the findings of Gbikeloluwa and Moo-young who reported the inhibitory effect of accumulated cellobiose and cellodextrin of low degree of polymerization<sup>13</sup>.

Effect of temperature on cellulase production was determined. It was observed that the 30°C temperature was proved best

temperature for the rice husks. Millet husks, wheat bran and banana peels. And 40<sup>0</sup>C was for the coir waste and saw dust.

The cellulase enzyme assay was conducted by two methods, CMCase assay method and FPase assay method and the determination of glucose molecule liberated from the substrate was done by using the Miller’s modified method of DNSA. 1 unit of cellulase activity was defined as the amount of enzyme which releases 10 ug of glucose in the 30 minutes under specified conditions.

**Table-1**  
**Growth of different selected fungal species on 1% CMC containing Potato dextrose agar plate after 48 hours**

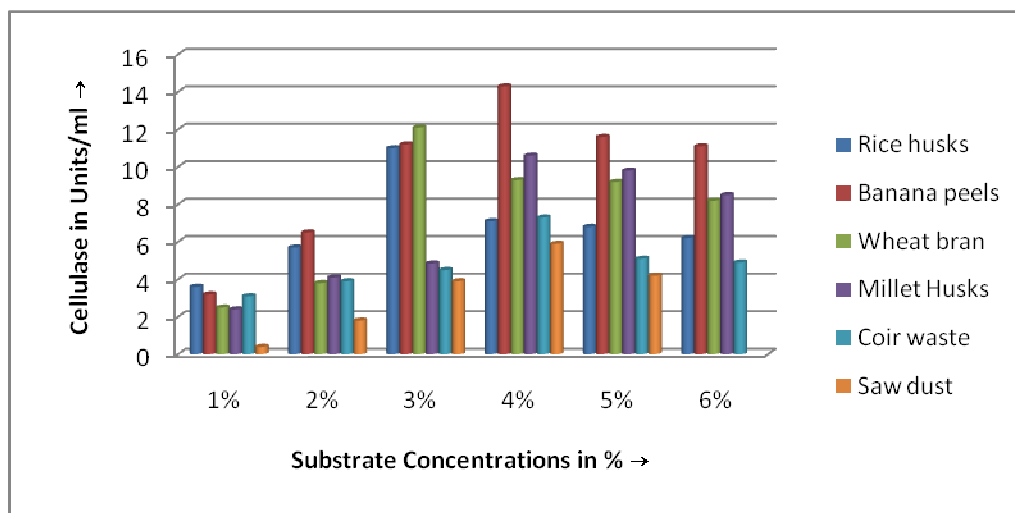
Sr. No.	Fungal Species	Diameter of Colony in mm
1	Aspergillus niger	34
2	Aspergillus oryzae	30
3	Aspergillus flavus	25
4	Penicillium chrysogenum	24
5	Fusarium moneliforme	18

**Table-2**  
**Effect of incubation period on Cellulase production assayed by CMCase assay**

Sr. No.	Days of Incubation →	II	III	IV	V	VI	VII
	Substrate ↓	Cellulase unit/ml	Cellulase unit/ml	Cellulase unit/ml	Cellulase unit/ml	Cellulase unit/ml	Cellulase unit/ml
1.	Rice husks	2.0	3.5	6.9	<b>10.9</b>	8.7	6.1
2.	Banana peels	2.5	4.8	8.2	<b>11.3</b>	10.1	6.2
3.	Wheat bran	2.9	3.4	6.6	<b>8.9</b>	5.1	3.6
4.	Millet Husks	2.2	3.5	6.5	<b>9.8</b>	6.1	4.8
5.	Coir waste	2.1	3.4	5.6	8.9	<b>9.6</b>	6.2
6.	Saw dust	1.8	2.6	4.2	6.3	<b>7.1</b>	4.2

**Table-3**  
**Effect of incubation period on Cellulase production assayed by FPase assay**

Sr. No.	Days of Incubation →	II	III	IV	V	VI	VII
	Substrate ↓	Cellulase unit/ml	Cellulase unit/ml	Cellulase unit/ml	Cellulase unit/ml	Cellulase unit/ml	Cellulase unit/ml
1.	Rice husks	2.4	3.9	7.2	12.2	9.4	5.6
2.	Banana peels	3.1	5.2	8.3	12.4	9.6	7.4
3.	Wheat bran	1.8	3.7	6.8	9.2	5.3	4.3
4.	Millet Husks	2.2	4.1	8.2	10.6	7.3	5.2
5.	Coir waste	1.6	3.6	5.3	8.2	9.2	7.1
6.	Saw dust	1.6	2.6	4.3	5.2	7.3	5.6



**Figure-1**  
**Effect of substrate concentration on cellulase activity in units / ml by FPase assay**

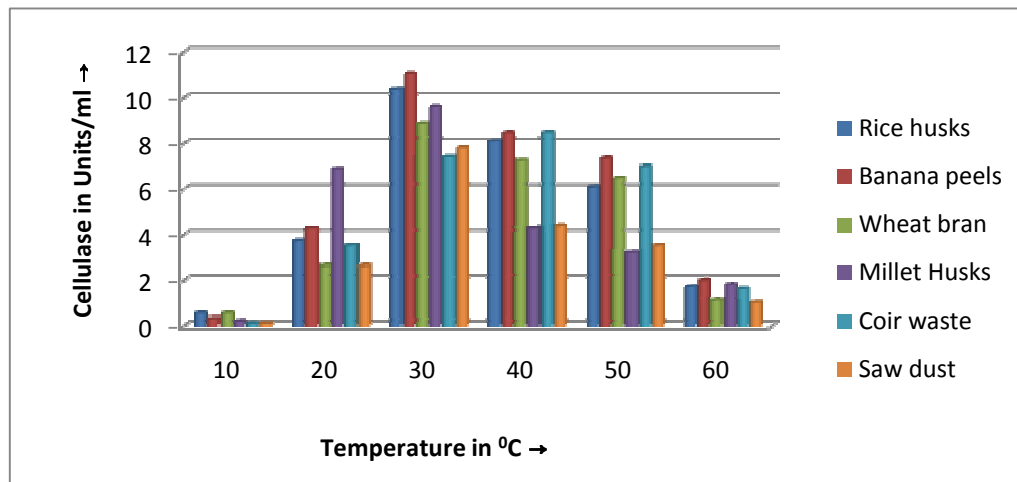


Figure-2  
Effect of Temperature on cellulase activity in units / ml by FPase assay

## Conclusion

On the basis of the above study it was concluded that, the selected fungal strains have the ability to degrade the agricultural wastes, out of the all fungal strains *Aspergillus niger* is the more efficient for the degradation of agricultural wastes. The uses of fungal strains for the enzyme productions have many advantages such as, the enzymes produced are normally extracellular, making easier for the extraction process. The used Dinitrosalicylic acid (DNS) method for the assessment of reducing sugars was suitable for routine analysis of reducing sugars. The present study was aimed at the condition optimization for the production of cellulase by using various agricultural wastes. And the Banana peels, Rice husks and Millet husks gave the higher production of the cellulase.

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