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# Optimization of cellulase production by *Aspergillus* species under solid state fermentation

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**Abstract**
Conversion of lignocellulosic waste to sugars is important application of microbial cellulases. In the present study, nine cellulose degrading fungi isolated from soil and agricultural waste such as corn husk, banana leaves etc. were screened for cellulase production. Various parameters were optimized for efficient production of cellulase enzymes using agricultural and plant residues by solid state fermentation for the isolate *Aspergillus* sp. Bl1. The isolate produced 0.94 $IUgds^{-1}$ FPase and 2.2 $IUgds^{-1}$ endoglucanase (CMC ase) after 72 h incubation at 37°C in optimized medium composed of banana leaves (C- source), tryptone as N-source, mineral salt solution having pH 5 and 67% moisture content. Other optimized parameters were; inoculum size of $7.35\times10^6$ spores /ml and distilled water (pH 7) as solvent for extraction of cellulases. The isolate produced 1.33 $IUgds^{-1}$ endoglucanase (CMCase) when cotton seed meal was used as N- source. Results indicate that agricultural waste such as banana leaves and cotton seed meal can be efficiently used for cellulase production by solid state fermentation using our isolate.

**Keywords:** cellulase, agricultural waste, solid state fermentation, Aspergillus species

## 1. Introduction
Cellulose is the most common organic polymer, representing about $1.5 \times 10^{12}$ tons of the total annual biomass production through photosynthesis, especially in the tropics, and is considered to be an almost inexhaustible source of raw material for different products [1]. The lignocellulosic plant waste can be converted to sugars by using cellulases. A number of approaches have been adopted, aiming towards reducing the cost of cellulase enzyme production; these have included the use of different lingo-cellulosic wastes. Lignocellulosic wastes such as sawdust, corn cob, bagasse, wheat straw, rice straw and wheat bran are examples of low-cost materials which have been successfully used as substrates for cellulase fermentation by fungi mainly *Aspergillus* sp. and *Trichoderma* sp [2, 3]. Solid state fermentation (SSF) requires less energy, involves easier downstream processing and is better and cost effective option for bioconversion of lignocellulosic waste [1, 4]. In this paper an attempt has been made to focus on the parameters which are relevant to enhance cellulase production using agricultural and plant residues under solid state fermentation by *Aspergillus* species.

## 2. Materials and Methods
### 2.1.1 Cultures
Nine cellulose degrading fungi isolated from agricultural waste like corn husk, banana leaves and soil were screened for cellulase production. A cellulose degrading *Aspergillus* sp. (Bl1) was isolated from agricultural waste and was selected for further studies.

### 2.1.2 Culture maintenance
Fungi were maintained on Potato Dextrose Agar (PDA) (g/l: Peeled potatoes 400, Dextrose; 20, Agar; 30, pH 4) and CMC agar slants (g/L: Carboxy Methyl Cellulose; 10, Sodium nitrate; 2, $KH_2PO_4$; 1, MgSO4.7H2O, 0.5, $FeSO_4$; 0.01 Agar; 30; pH 4).

### 2.2 Cellulosic Substrates
Different types of agricultural and plant residues (corn husk, rice husk, sorghum husk, banana leaves, saw dust, wood filings) were collected from environ of Pune, India. The raw material was dried, crushed and sieved through 0.01 mm sieve size.

### 2.3 Methods

#### 2.3.1 Screening of cellulolytic ability in liquid medium

Cellulolytic ability of nine fungal isolate was first checked using cellulose broth (g/L: Cellulose, 10; Sodium nitrate, 2; $KH_2PO_4$, 1; $MgSO_4.7H_2O$, 0.5; $FeSO_4$, 0.01; Agar, 30 and pH 4). Inoculum was prepared by inoculating one ml of spore suspension ($10^6$ - $10^7$spores/ml) from seven days old fungal culture in 5 ml of sterile cellulose broth followed by incubation in shaker incubator at 30 °C at 180 rpm for 24 hours. 10% (v/v) inoculum was inoculated in 50 ml of cellulose broth and the flasks were incubated on shaker at 30 °C at 180 rpm.

Samples were removed after every 24 hours till 96 hours, centrifuged at 10000 rpm for 10 minutes at 4 °C. The cellulolytic ability of culture supernatant was assayed as described below.

#### 2.3.2 Cellulase assay

Cellulase activity was determined using 1% (w/v) CMC as a substrate. A reaction mixture was composed of 0.45 ml of 1% (w/v) CMC in 0.1 M acetate buffer (pH 4), 0.15 ml of acetate buffer and 0.4 ml of culture supernatant, was incubated at 37 °C for 30 min. The reducing sugar was determined by Nelson Somogyi's method [5] using glucose as standard. Individual blank was kept for every culture supernatant.

#### 2.3.3 Enzyme production by Solid State Fermentation

Erlenmeyer flask (250ml) with 2 g of powdered substrate (Carbon source) was used for optimization of various parameters. Substrate was sterilized at 121 °C for 30 minutes for 3 consecutive days before use. Substrate was moistened with 2 ml of sterile salt solution (g/L- $NH_4Cl$, 10; $KH_2PO_4$, 3; $MgSO_4.7H2O$, 0.05; $CaCl_2.2H_2O$, 0.05) and inoculated with 1 ml of spore suspension and mixed thoroughly to ensure uniform distribution. The flasks were incubated at 30°C for 72 hours in static condition. The enzyme was extracted by adding 20 ml of 0.1 M acetate buffer (pH 4) and keeping the flask at 180 rpm for one hour on a shaker. The culture supernatant was collected by filtering through muslin cloth and cellulase assay was carried out as mentioned before.

#### 2.3.4. Optimization of different physiochemical parameters

The pH of the medium was adjusted by adjusting the pH of the salt solution and using 1N HCl and 1N NaOH before autoclaving. Salt solutions with pH 4, 5 and 6 were used. Temperatures of 25 °C, 30 °C and 37 °C were selected for optimization. Moisture content was adjusted by varying the volume of salt solution without changing the concentration of salt solution. Moisture content was adjusted to different values in the range 50% to 70%.

Inoculum size was optimized by adjusting different optical densities (O. D.) within the range of 0.4 to 1.8 on colorimeter and determining the corresponding spore count by Neubauer chamber. Different extracting buffers, citrate buffer (pH 5.5), citrate buffer (pH 4), phosphate buffer (pH 7), acetate buffer (pH 5), acetate buffer (pH 4) and distilled water were used for enzyme extraction.

Organic nitrogen sources used for optimization were malt extract, cotton seed meal, soya bean meal, beef extract, peptone, yeast extract and tryptone while inorganic were $NaNO_3$, $KNO_3$, urea and $(NH_4)_2SO_4$. During media preparation, $NH_4Cl$ from the salt solution was replaced on equal weight basis by respective nitrogen sources.

Crude carbon sources like corn husk, banana leaves, saw dust, wood filings, rice husk, and sorghum husk were used.

#### 2.3.5 Substrate specificity of cellulase

The substrate specificity was studied using different sources of cellulose to check the enzyme activity

**1) Absorbent cotton**

100 mg of cotton was moistened with 0.1 ml of acetate buffer (0.1 M pH-4), mixed with 2ml of enzyme and incubated at 50 °C for 24 hours. The reducing sugar produced in the supernatant was estimated the by Nelson-Somogyi's method [5].

**2) Whatman paper no. 1: FPase activity [6]**

For FPase activity, 50 mg of Whatman no. 1 filter paper was used as a substrate. Crude culture filtrate was used as an enzyme sample. 1×6 cm piece of Whatman paper (50 mg), 1ml of 0.1 M acetate buffer (pH-4) and 0.5 ml of enzyme were incubated for 1hour at 37 °C. The reducing sugar was determined by Nelson Somogyi's method using glucose as standard. Individual blank was kept for every culture supernatant. Filter paper activity is a combined assay for Endoglucanase (Cx) and Exoglucanase (Ci) cellulase.

**3) Cellulose powder**

100 mg cellulose powder 5ml of 0.1 M acetate buffer (pH 4) and 1 ml of culture supernatant was incubated for 1hour at 37 °C. The reducing sugar was determined by Nelson Somogyi's method.

**4) CMC (Carboxy Methyl Cellulose) [7]**

The reaction mixture was composed of 0.45 ml of 1 % (w/v) CMC in 0.1 M acetate buffer (pH 4), 0.15 ml of acetate buffer and 0.4 ml of culture supernatant, was incubated at 37 °C for 30 min. The reducing sugar was determined by Nelson Somogyi's method [5] using glucose as standard.

### 3. Results and discussion

#### 3.1 Cellulase production in liquid medium

In this study, cellulolytic ability of nine cellulose degrading fungi was checked in a liquid medium. Among them, fungal isolate Bl1 gave highest cellulase yield of and 0.0049 IU/ml after 72 hours of incubation (Table 1).

**Table 1:** Cellulase yield of fungal isolates in liquid medium

| Isolate | Cellulase yield (IU/ml) | | | |
|---|---|---|---|---|
| | 24 h | 48 h | 72 h | 96 h |
| C | 0.0001 | 0.0002 | 0.0004 | - |
| G | 0.0002 | 0.0003 | 0.00031 | 0.0032 |
| Co1 | 0.0017 | 0.0023 | 0.0025 | 0.0023 |
| Light Green | - | 0.0003 | 0.00021 | 0.00022 |
| i5 | 0.0001 | 0.00015 | 0.00018 | 0.00018 |
| Un2 | 0.0002 | 0.0002 | 0.0002 | - |
| Bl1 | 0.002 | 0.0034 | 0.0049 | 0.0045 |
| le1 | 0.0024 | 0.0031 | 0.0036 | - |
| *A.niger* | 0.0001 | 0.0005 | 0.0002 | 0.0003 |

#### 3.2 Optimization of pH

For isolate Bl1 pH 5 (Fig. 1) was found to be optimum for CMCase production with activity 0.1IU/g-ds. P.B. Acharya *et al.* [8] and Gupta *et al.* [3] have reported the maximum cellulase production at pH 4 and 4.5 respectively by *A. niger* using SSF.

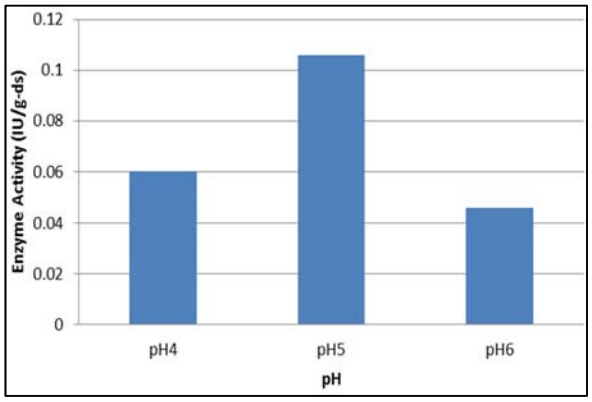


**Fig 1:** Optimization of growth pH for cellulase production.

### 3.3 Optimization of temperature

The optimum temperature was found to be 37 °C with activity 0.2IU/g-ds (Fig 2). Gupta *et al.* [3] reported maximum activity at 32 °C

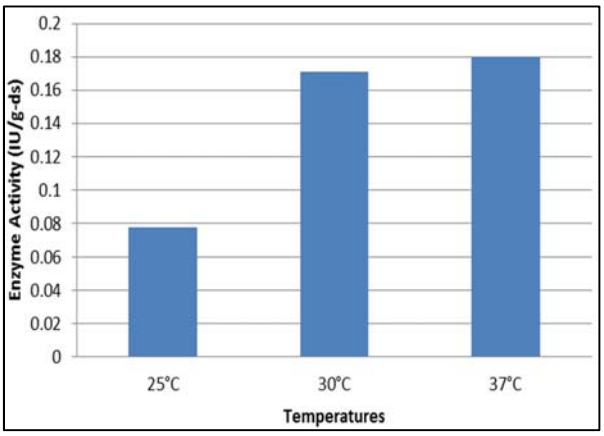


**Fig 2:** Optimization of growth temperature for cellulase production.

### 3.4 Optimization for moisture content

For Bl1 isolate, the optimum moisture content was 67 % and activity was 0.21 IU/g-ds (Fig 3). This result was comparable with 57% moisture content (0.20 IU/g-ds). Sherief *et al.* [2] and Maurya *et al.* [9] observed that 75% and 70% moisture content was optimum for *A. fumigatus* and *Trichoderma reesei* respectively.

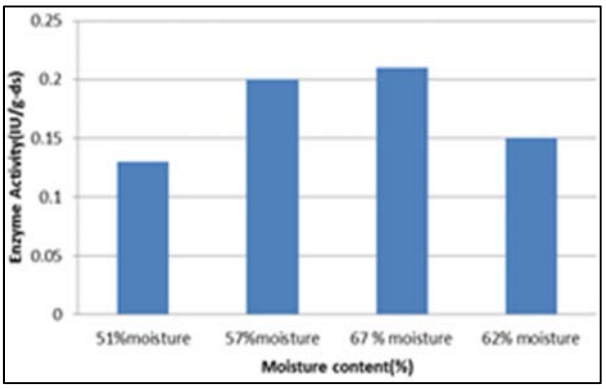


**Fig 3:** Optimization of moisture content for cellulase production.

### 3.5 Optimization for inoculum size

For both isolates, different O.D. values were selected and corresponding number of spores/ml were calculated using Neubauer chamber.

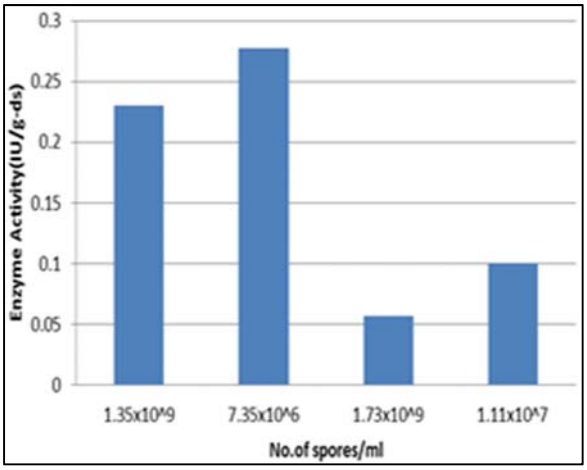


**Fig 4:** Optimization of inoculum size for cellulase production.

For Bl1 isolate, 7.35× $10^6$ spores/ml (O.D = 0.4) was found to be optimum inoculum with activity 0.27IU/g-ds (Fig 4). Abo state *et al.* [10] have reported that best incolum size was 0.5 ml 0f $2\times10^7$ spores/ml for *Aspergillus tereus* and *Aspergillus flavus* for cellulose production by SSF using wheat straw as substrate.

### 3.6 Optimization for extracting buffer

Maximum enzyme activity obtained was (0.30IU/g-ds) when the distilled water was used for extraction of enzyme (Fig. 5). The yield obtained with citrate buffer (pH5.5) was 20% lesser (0.25IU/g-ds). Gautam *et al.* [11] have used citrate acetate buffer (pH 5) for extraction of cellulase.

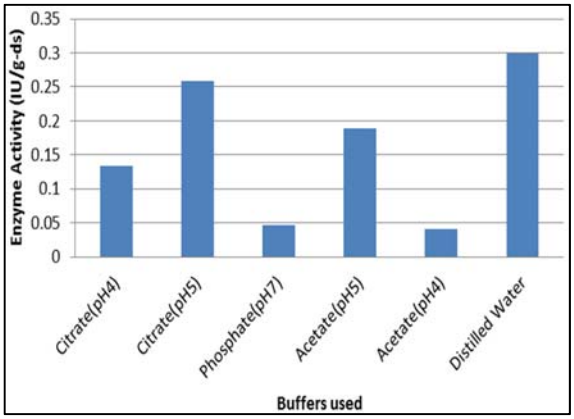


**Fig 5:** Optimization of extraction buffer.

### 3.7 Optimization of Nitrogen sources

Tryptone was found to be the optimum N-source with activity 2 IU/g-ds for the isolate Bl1 (Fig. 6). Chundakkadu Krishna [12] reported $(NH_4)_2SO_4$ and $NaNO_3$ as optimum nitrogen sources but for isolate Bl1 $(NH_4)_2SO_4$ gave 133 fold less activity as compared to Tryptone. Gautam *et al.* [11] have reported peptone and yeast extract as best nitrogen sources for cellulose production.

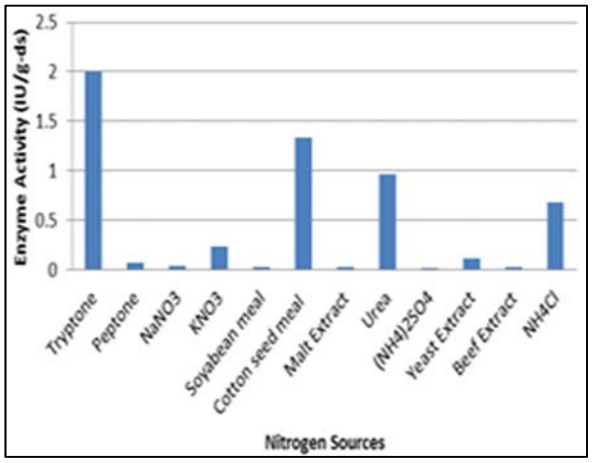


**Fig 6:** Optimization of Nitrogen source for cellulase production

### 3.8 Optimization of carbon sources

Among all the C-sources tested the maximum yield was found to be 2.2 IU/g-ds with banana leaves as C-source. It was 20 fold greater compared with wood filings. Chundakkuadu Krishna (1999) [12] discussed the use of various C-sources on the basis of which the substrates were selected. Immanuel *et al.* [13] have reported use of coir waste and saw dust as substrates for cellulase production by submerged fermentation.

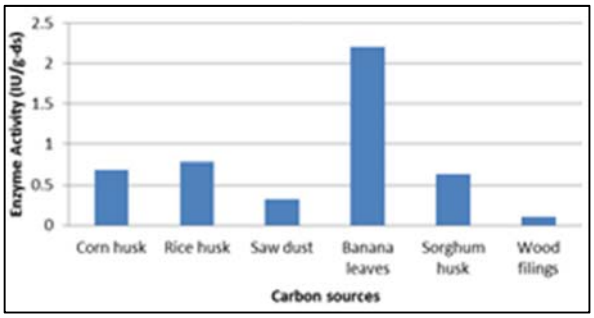


**Fig 7:** Optimization of carbon source for cellulase production.

### 3.9. Cellulase activity with different substrates

For Bl1 isolate, the maximum endoglucanase activity (2.2 IU/g-ds) was found with CMC as a substrate. FPase activity of 0.94 $IUgds^{-1}$ was obtained with Whatman filter paper. Sherief *et al.* [2] observed that Aspergillus fumigatus produced 21 fold more endoglucanase as compared to FPase, whereas for Aspergillus sp. We found only 2 fold difference in the activities.

## 4. Conclusions

In the present work, nine cellulose degrading fungi which were isolated from different samples were checked for their celluolytic ability in cellulose broth. Out of those fungal isolates, Bl1 and which gave maximum enzyme activity in liquid medium, was chosen for optimization of cellulase production by using Solid State Fermentation.

The optimized medium for Bl1 isolate was composed of banana leaves, tryptone as N-source, pH 5 of mineral salt solution, 67% moisture content. The optimized inoculum size was found to be $7.35\times10^6$ spores/ml and optimum temperature 37 °C. Distilled water (pH 7) was found to be optimum solvent for extraction of cellulase. The use of CMC as optimum substrate gave maximum activity.

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