Study on the Cellulase Activity of Fungi by Solid State Fermentation Using Cellulosic Wastes

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Abstract: The present study aims to use of fungi for the degradation of cellulosic wastes like Waste Paper, Cotton ginning, Wheat Bran, Sugarcane bagasse and Cellulose using solid state fermentation for cellulase production. Total 34 isolates were obtained from various sources by the primary screening technique from which 14 isolates were showing higher cellulase activity. Potential isolates were obtained from wood furnishing region and paper industry waste. Different fungal strains were tested to find their ability to produce cellulase, which catalyses the degradation of cellulose, which is a linear polysaccharide made of glucose subunits linked by 1-4 glycosidic bonds. Selected 14 fungi strains were inoculated on different agriculture waste for the production of cellulase enzyme. Among these, wheat bran gave maximum zone of hydrolysis of carboxy-methyl cellulose and shown higher activities of the cellulase, which were determined by Filter paper assay (FPA) and Carboxy-methyl cellulase assay (CMCase). Maximum production of cellulolytic enzymes was found in wheat bran. A4, A5, A7, A8 and A10 produced higher endoglucanase activity 5.36 U/g, 5.32 U/g, 7.53 U/g, 6.53 U/g, 5.26 U/g respectively as well as higher FPase activity 2.45 U/g, 0.95 U/g, 1.86 U/g, 1.98 U/g, 1.06 U/g respectively.

Keywords: Fungi, Solid stat fermentation, FPase, CMCase, Cellulosic wastes

1. Introduction

Agricultural and industrial wastes are among the major causes of environmental pollution. Their conversion into useful products may ameliorate the problems they causes. These wastes which include mainly leaves, straws, cereals, corncobs etc., in many countries, these materials are generally used as animal feeds. Cotton waste and discarded paper also used as a cellulosic waste. A huge quantity of cotton gin waste is generated in cotton mills. Globally, India is the second largest cotton producing country which produces undesired cotton gin waste (Sahu S. and Pramanik K., 2015). A huge amount of these materials are left on farmlands to be decomposed by microorganisms such as Bacteria and fungi (Jadhavet et al., 2013).

Cellulose is commonly degraded by an enzyme called cellulase. Fungi are the main cellulosic producing microorganisms, though a few bacteria and actinomycetes have also been reported to yield cellulase activity. For complete hydrolysis of cellulose to glucose, cellulose systems must contain endo-1, 4-glucan (1, 4 - D-glucanohydrolase’ EC 3.2.1.4), exo -1, 4 -glucan (1, 4 - D-cellobiohydrolase’ EC 3.2.1.9) and -glucosidase (- D -glucohydrolase’ EC 3.2.1.91) or celllobiase. Thus the hydrolysis of cellulose is completed by the synergistic action of endo- and exoglucanases(Fadelet et al., 2000).

The enzymes of industrial importance have traditionally been produced in submerged fermentation (SmF) because of the ease of handling and good control of environmental factors such as temperature, aeration, agitation and pH (Singh et al., 2007). However, solid state fermentation (SSF) techniques are better adapted to enhance the yield, which reduces the cost of enzyme production because of the ability of filamentous fungi to grow well on solid substrates (Ghildyalet al., 1985; Pandey, 1992; Hui et al., 2010). The other advantages of SSF include maximum productivity; ease of technique; low capital investment, low energy requirement and less water output, better product recovery and lack of foam build up and reported to be most appropriate process for developing countries (Zeng and Chen, 2009 and Souza and Magalhaes, 2010).

Cellulases have enormous potential in industries and are used in food, beverages, textile, laundry, paper, waste management, medical/pharmaceutical industry, protoplast production, genetic engineering and pollution treatment (Jahangeer et al., 2005) and pulp industries etc.

2. Materials and Methods

2.1 Isolation and Screening of Cellulose-Degrading Fungi

Samples including paper industry waste, municipal waste, sugarcane farm, garden, and wood furnishing were collected from different site of Gandhinagar and Kadi in sterile polythene bags. Isolation and Screening of cellulolytic fungal species was done on Carboxyl agar medium. The presence of cellulase was tested using the media proposed by Hart et al., (2002). The individual microorganism grown on basal salt media supplemented with 1% Carboxy methylcellulose (CMC) used as carbon source. The purified cultures were inoculated in the center with almost equal amounts and incubated at 37°C until substantial growth was recorded. The petri plates were kept at 50°C for 30 min. Plates were flooded with 1% Iodine solution and allowed it to stand for 5-10 minutes. The clear zone was observed around the colony due to cellulose hydrolysis.

2.2 Characterization and Identification of fungal Isolates

Fungal colonies were isolated form different samples for cellulase producing microorganisms by serial dilution method. 100µl of samples diluted up to 10⁻³ dilutions, were spread on respective solidified PDA plates. The inoculated
petri plates were incubated at 30°C for 48 hours. The isolates were inoculated on sterile PDA plates by four flame method and incubated at 30°C for 48 hours in order to obtain pure cultures. Colony characteristics, morphological characteristics and microscopic examinations of the various isolates were determined and the reproductive and vegetative structures were also studied (Devenanath et al. 2007).

2.3 Substrate preparation for enzyme production

Five different types of Cellulosic waste based medium was used for production of cellulytic enzymes. The Cellulose, Wheat bran, Sugarcane bagasse, Cotton waste and discarded paper were used as substrate for the production of cellulase. Cotton Waste and discarded paper were pretreated with 3% H2SO4. This 2.0 gm of substrates were supplemented to the basal medium. The composition of Basal medium includes KH2PO4: 2gm, (NH4)2PO4: 1.4 gm, Urea: 0.3 gm, CaCl2.2H2O: 0.3 gm, MgSO4: 0.3 gm, Peptone: 1.0 gm, FeSO4.7H2O: 5.0 mg, MnSO4.7H2O: 1.6 mg, ZnSO4.7H2O: 1.4 mg, CoCl2.6H2O: 2 mg, Tween 80: 0.2% (v/v), D/W 1000 ml and pH: 5.5. Mandel and Resse (1957)

2.4 Experimental design of solid state fermentation (SSF)

Inoculum preparation was carried out using Sabouraud dextrose broth (150 ml), prepared in 500ml Erlenmeyer flask and autoclaved at 15 lbs for 15 min. The medium was inoculated with isolated fungus of 3 mycelial disc (7 mm diameter) punched out from the edges of its 8 days old colonies from Petri plates. The flasks were incubated at 30 ± 2 °C for 72 hrs.

The fungus was inoculated into substrates flasks for enzyme production. Enzymes were extracted from substrate flasks by addition of 5 ml of cold 0.05 M acetate buffer (pH 4.8). The homogenate was filtered through muslin cloth and the filtrate was centrifuged at 5000 rpm at 4°C for 15 min. The supernatant was analyzed for carboxyl methyl cellulase (CMCase) and filter paper activity (FPase).

2.5 Enzyme Assay

2.5.1 Filter paper assay (FPase)

Filter paper activity of the culture filtrates was determined according to the method of Mendels and Weber (1969). Whatman filter paper strips containing 50 mg (1cm X 6cm) weight was inoculated in 1 ml of 0.05 M sodium acetate buffer (pH 4.8) and kept at 50 °C in a water bath. Suitable aliquots of enzyme source were added to the above mixture and incubated for 60 minutes at 50°C. After incubation, the liberated reducing sugar was estimated by the addition of 3, 5-dinitrosalicylic acid (DNS). The color developed in tubes were measured at 540 nm using spectrophotometer. Appropriate control without enzyme was simultaneously run. Activity of cellulase was expressed in filter paper units. One filter paper unit (FPU) was defined as the amount of enzyme releasing 1 mole of reducing sugar from filter paper /ml /min.

2.5.2 Endoglucanases enzyme assay (CMCase): Endoglucanase activity of Carboxy methyl cellulase (CMCase) was carried out as described by Ghosh (1987). The reaction mixture of 0.5 ml enzyme, 0.5 ml of 1% carboxy methyl cellulose in 0.05 sodium acetate buffer (pH 5.0) and 1ml DNSA were incubated at 50 °C in a water bath for 20 minute. The release of glucose due to the enzyme activity was assayed by 3, 5 Dinitro salicylic acid measured at 540 nm using spectrophotometer (Miller, 1959).

3. Results and Discussion

In this investigation, we have navigated the isolation of cellulytic fungi from paper industry waste, municipal waste, sugarcane farm, garden, and wood furnishing. Samples were collected from different site of Gandhinagar and Kadi. Total 34 different isolates were obtained by the primary screening technique from which 14 isolates were showing higher cellulase activity. Potential isolates were obtained from wood furnishing region and paper industry waste shown- in table no 1. In the Primary screening, cellulase activity was determine by dith method as well as spot inoculation method. Cellulase activity was measured after 72 hrs of incubation.

The fungal strains were tested for their cellulytic ability on plate clearing assay by using Carboxy methyl cellulose and iodine. Formation of clear zone diameter on CMC agar were measured in mm. 14 out of 34 isolates were selected based on cellulose hydrolysis ranging from 5 mm to 15mm diameter of clear zone shown in Figure 1. Morphological, Microscopic observation and cellulose hydrolysis zone were shown in table 1.

<p>| Table 1: Colony morphology and microscopic observation of selected culture |
|--------------------------|-------------------|--|--|</p>
<table>
<thead>
<tr>
<th>Isolated Culture</th>
<th>Isolation from</th>
<th>Colony Morphology</th>
<th>Cellulase Zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Soil</td>
<td>White to green, dark green spore</td>
<td>7</td>
</tr>
<tr>
<td>A2</td>
<td>Cotton waste</td>
<td>White to green, dark green spore</td>
<td>14</td>
</tr>
<tr>
<td>A3</td>
<td>Cotton waste</td>
<td>Green to black</td>
<td>12</td>
</tr>
<tr>
<td>A4</td>
<td>Vegetable waste</td>
<td>Green to Black, Black mycelium</td>
<td>21</td>
</tr>
<tr>
<td>A5</td>
<td>Wood</td>
<td>White to Green, Green spore</td>
<td>18</td>
</tr>
<tr>
<td>A6</td>
<td>Cotton waste</td>
<td>White to green, dark green spore</td>
<td>13</td>
</tr>
<tr>
<td>A7</td>
<td>Soil</td>
<td>Green to Black</td>
<td>21</td>
</tr>
<tr>
<td>A8</td>
<td>Plant</td>
<td>White mycelium, Greyish green</td>
<td>12</td>
</tr>
<tr>
<td>A9</td>
<td>Soil</td>
<td>White to green, dark green spore</td>
<td>12</td>
</tr>
<tr>
<td>A10</td>
<td>Paper waste</td>
<td>White to Green</td>
<td>18</td>
</tr>
<tr>
<td>A11</td>
<td>Soil</td>
<td>White to light Green, light and dark green mycelium</td>
<td>25</td>
</tr>
<tr>
<td>A12</td>
<td>Plant</td>
<td>White to light Green</td>
<td>22</td>
</tr>
<tr>
<td>A13</td>
<td>Plant</td>
<td>White to green, dark green spore</td>
<td>13</td>
</tr>
<tr>
<td>A14</td>
<td>Plant</td>
<td>White to light green, light green mycelium</td>
<td>8</td>
</tr>
</tbody>
</table>
Experiment was designed using solid state fermentation for optimum cellulase production. Among five different cellulosic substrates, four were different lignocellulosic wastes (waste paper, cotton, wheat bran, sugar cane bagasse) and fifth was pure cellulose. About $1 \times 10^7$ spores of different 14 fungi were inoculated in the solid state substrate flask. The enzyme activity was analysed only after the 2nd day of incubation to allow optimal fungal growth. It was reported earlier that the enzyme production by the fungi started after a lag period of 24 hr or more, and the activities reached to optimal levels within 3-5 days of incubation (Gomes I et al., 2006). FPase activity and CMCase activity of each culture from different substrates were carried out at the interval of 24 hrs at 30°C.

![Figure 2: Inoculum for development](image1)

![Figure 3: Solid State Fermentation for cellulase production](image2)

Maximum FPase activity was achieved after 3 days of incubation by utilizing different cellulosic substrates like wheat bran, sugarcane bagasse, cotton waste, discarded paper, and cellulose. But both CMCase and FPase activity was decline after the 5th day of incubation, which is shown in all graphs.

FPase and CMCase activity on paper waste of all 14 fungal cultures were shown in Figure 4. After 96 hrs. of incubation, A4, A5, A7, A8 and A10 cultures gave higher FPase activity 1.22 U/g, 0.94 U/g, 1.0 U/g, 0.73 U/g, and 1.05 U/g respectively compared to other fungal cultures. While CMCase activity of A4, A7 and A8 cultures were 4.21 U/g, 3.64 U/g, and 5.21 U/g respectively after 24 hrs. These show that cultures can be utilized for degradation of cotton ginning waste which was the major waste of India.

![Figure 4: FPase and CMCase activity on Paper](image3)

![Figure 5: FPase and CMCase activity on Cotton](image4)

FPase and CMCase activity on cotton waste of all 14 fungal cultures were shown in Figure 5. After 96 hrs. of incubation, A4, A5, A7, A8, and A10 cultures gave higher FPase activity 1.22 U/g, 0.94 U/g, 1.0 U/g, 0.73 U/g, and 1.05 U/g respectively compared to other fungal cultures. While CMCase activity of A4, A7, and A8 cultures were 4.21 U/g, 3.64 U/g, and 5.21 U/g respectively after 24 hrs. These show that cultures can be utilized for degradation of cotton ginning waste, which was the major waste of India.
FPase and CMCase activity on Wheat Bran of all 14 fungal culture were shown in Figure 6. After 72 hrs. of incubation, A4, A7, A8, A10 and A12 cultures gave higher FPase activity 2.45 U/g, 1.86 U/g, 1.98 U/g, 1.05 U/g, and 1.14 U/g respectively compare to other fungal cultures. While CMCase activity of A4, A7, A8 and A10 cultures were 5.36 U/g, 7.53 U/g, 6.53 U/g and 5.26 U/g respectively after 72 hrs. This higher activity indicate that wheat bran can be used as cheapest alternative source for cellulase production.

Figure 6: FPase and CMCase activity on Wheat bran

FPase and CMCase activity on Sugarcane of all 14 fungal culture were shown in Figure 7. After 72 hrs. of incubation A4, A5 and A7 cultures gave higher FPase activity 0.53 U/g, 0.50 U/g and 0.53 U/g respectively and A8 gave 1.35 U/g after 120 hours of incubation compare to other fungal cultures. While CMCase activity of A4, A7, and A8 cultures were 5.59 U/g, 7.47 U/g and 7.56 U/g respectively after 120 hrs. and A5 gave 4.46 U/g after 72 hrs. of incubation.

Figure 7: FPase and CMCase activity on Sugarcane bagasse

FPase and CMCase activity on Cellulose of all 14 fungal culture were shown in Figure 8. After 72 hrs. of incubation A4, A5, A7, A8 and A10 cultures gave higher FPase activity 1.15 U/g, 1.24 U/g, 1.46 U/g, 1.50 U/g and 1.02 U/g respectively and A14 gave 1.5 U/g after 120 hours of incubation compare to other fungal cultures. While CMCase activity of A4, A7, and A8 cultures were 4.61 U/g, 7.47 U/g and 7.56 U/g respectively after 120 hrs. and A5 gave 4.46 U/g after 72 hrs. of incubation.

Figure 8: FPase and CMCase activity on Cellulose
Among five different cellulosic substrates waste paper, cotton, wheat bran, sugar cane bagasse and cellulose tested in solid state fermentation (SSF) by different 14 fungi, wheat bran yielded maximum enzyme activity of FPase and endoglucanase after 96 hours of growth. A4, A5, A7, A8 and A10 produced high endoglucanase 5.36 U/g, 5.32 U/g, 7.53 U/g, 6.53 U/g, 5.26 U/g and FPase 2.45 U/g, 0.95 U/g, 1.86 U/g, 1.98 U/g, 1.06 U/g respectively.

Among all 14 selected fungal culture A7 and A8 shows high FPase and CMCase activity in all cellulosic substrates which is shown in Figure 9. From the present study it was conclude that, among five cellulosic substrates Wheat Bran gave higher FPase and cellulase activity by using A8 culture yielding 1.98U/g and 7.53 U/g during 3 day incubation. Haque et al., (1989, 1990, and 1991) and Subhosh Chandra et al., 2007 also studied solid substrate fermentation by different fungi using different cellulosic biomass and they also found maximum enzymes production using wheat bran.

4. Conclusion

Cellulosic wastes are most abundant renewable resources in the biosphere which have been shown to be used in the production of valuable products by microorganisms specially fungi. Total 34 fungi were isolated from different Agriculture waste. According to clear hydrolysis zone of cellulose, 14 wild type strains have been selected for cellulase production. Production of cellulases on Cellulosic wastes under Solid state fermentation was studied by different 14 strain of fungal have the potential of converting cellulose in a single step fermentation process. Among different agriculture waste wheat bran was show higher activities of the cellulase were determined by Filter paper assay (FPA) and Carboxy-methly cellulase assay (CMCase) assay. Cellulase production with A7 and A8 were maxium on 3 day of incubation and in presence of substrates (Wheat bran) at 37°C. These two cultures were producing higher cellulase which can be used for future bioethanol prospects.

5. Future Scope

The isolated microorganism and selected lignocellulosic waste can be used for further bioethanol prospects.

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7. References


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