PRODUCTION OF FUNGAL CELLULASE FROM AGRICULTURE SOIL ISOLATES USING CMC AS SUBSTRATE UNDER SUBMERGE FERMENTATION TECHNOLOGY

P.K. Chouhan*

Faculty, Department of Microbiology, Govt. Arts and Science PG College, Ratlam, Madhya Pradesh, India.

ABSTRACT

Cellulase is an enzyme has great industrial importance. It is used as cellulose hydrolyzing enzyme and produces small sugar components i.e. glucose, cellobiose and other oligomers. In the present study we tried to isolate the fungal organism, *Rhizopus* and *Aspergillus*, from agriculture soil since the fungi have been always the best effective microorganism source for cellulose production. The enzyme production was increased with incubation time during submerge fermentation process by both fungal isolates using carboxy methyl cellulose as substrate whereas more enzyme activity was shows by *Aspergillus* spp than *Rhizopus* spp.

**KEY WORDS:** Cellulase, *Aspergillus*, *Rhizopus*, Submerge Fermentation Technology and Incubation time.

INTRODUCTION

Cellulolytic enzyme play an important role in biodegradation processes where plant lignocellulosic material is efficiently degraded by cellulolytic fungi and bacteria. In industry, cellulolytic enzymes have found novel applications in the production and processing of chemicals, foods and manufactured goods such as paper, rayon and cellophane. Cellulases are a group of enzymes that break down cellulose into glucose monomers. Cellulase is produced by several microorganisms, commonly by bacteria and fungi. Bacterial and fungal cellulases are traditionally separated into three classes: Endoglucanases, exoglucanases and β-glucosidases based on the ability to degrade carboxy methylated...
cellulose (CMC) \cite{5}. The production of this enzyme has increased significantly and today its production is around 20% of all the enzymes produced in the world. Jahangeer et al. \cite{6} isolate fungal strains from various environments for cellulose activity and found that \textit{A. niger} shows highest enzyme activity in their culture filtrate. \textit{A. fumigatus} was studied for lignocellulosic substrate and found that Fpase and Cmcase activity was highest at pH 6.5 and 32\(^\circ\)C temperature \cite{7}.

Fungi and bacteria are the main natural agents that degrade cellulose and their population includes aerobic and anaerobic mesophilic bacteria, filamentous fungi, thermophilic and alkaliphilic bacteria, actinomycetes and certain protozoa \cite{8, 9}. However, fungi are well known agents that decomposed organic matter, in general, and of cellululosic substrate in particular \cite{10}. Cellulase producing fungi includes genera \textit{Trichoderma, Penicillium, Botrytis Neurospora, Aspergillus niger, Aspergillus terreus, Rhizopus stolonifer} and \textit{Aspergillus} \cite{11, 12, 13}. Fungi are capable of decomposing cellulose, hemicelluloses and lignin in plants by secreting enzymes \cite{14}.

Most of the commercial cellulase have been produced from the \textit{Trichoderma, Penicillium} and \textit{Aspergillus} genera \cite{15, 16}. Microorganisms excreting cellulase play an important role in the nature due to their ability to decompose lignocellulosic residues and established a key link in the carbon cycle \cite{17}. Fungal extracellular enzymes play a essential role in the degradation of substrates such as lignocelluloses \cite{18}. Cellulases produced by the filamentous fungi, yeasts and bacteria are implicated in the degradation of cellulose fibers to soluble sugars such as glucose, cellobiose and other oligomers. Economically cellulase convert cellulytic material to glucose would be significant for industry and their potential applications include the production of fruit juice, food, beverages and also used in textile, laundry, paper and pulp industries \cite{19, 20}.

The objects of the present work is to identify the fungal strains i.e. \textit{Aspergillus} and \textit{Rhizopus} as have potential for cellulose production, and comparatively enzyme production during varying incubation time by using CMC as substrate for submerge fermentation technology.

**MATERIAL AND METHODS**

**SOIL SAMPLE COLLECTION**

Ten to twelve soil samples were collected from agricultural crop fields towards Joara and Sailana road, Ratlam Madhya Pradesh, India and were mix to form one composite sample.
Soil sample was crushed using Mortar and Pestle to clean by removing rock particles and plant debris.

**ISOLATION OF FUNGI**

Fungi were isolated from soil by serial dilution technique using 10g soil [21, 22]. Soil was dissolved in 0.85% saline water in Erlenmeyer conical flask (500ml capacity) having 95ml saline water and beads then shaken to mix soil properly, mark as 10⁻¹ dilution. Obtain 10⁻³ dilution by serially transferred 10ml soil sample from 10⁻¹ dilution to conical flask having 90ml saline water. 0.1ml sample was transferred from 10⁻³ dilution to sterilize Petri dish and poured melted Potato dextrose agar (PDA) medium. Plate was shaking gently to mix sample and medium then left at room temperature to solidify medium. The solidified plate was incubated at 30°C for 5 days. After that plate was used for observation.

After incubation period, few spores were taken by wire loop and streaked on PDA medium plate by zigzag method and incubated at 30°C for 5 day. Colonies were observed by light microscope using 10 and 40X objective lens. Pure colonies were transferred to PDA plate and used for identification.

**IDENTIFICATION OF FUNGI**

Fungal culture was stain with Cotton blue stain and lacto phenol and covered with glass cover slip then observed in light microscope using low power (10X) and high power (40X) objective lens. Morphological characteristics of fungal culture were identified using laboratory manual for introductory mycology [23, 24, 25].

**FUNGAL SCREENING**

The isolated fungal cultures were screened for their ability to produce cellulase on selective media contained NaNO₃– 2g, K₂HPO₄– 1g, MgSO₄.7H₂O– 0.5g, KCl– 0.5g, Carboxy methyl cellulose sodium salt– 2g, Agar agar- 17g and Distilled water- 1000ml. pH of the medium was adjusted to 5.0. After autoclaving at 121°C and 15lb pressure, the medium was poured into Petri plates and allowed to solidify then inoculated with fungal culture. The plates were incubated at room temperature (30°C) for five days to allow fungal growth. After incubation, 10ml of 1% Congo red staining solution was added to the plate and was shaken for 15min. The Congo red staining solution was discarded and added 10ml of 1N NaCl then again shaken to de-stain plate for 15min. Finally 1N NaCl was discarded and the stained plates
were analyzed by observing the formation of clear zone around the fungal colonies. The high zone of clearance showing fungal isolates was used for cellulose production.

FERMENTATION PRODUCTION MEDIA
The submerge fermentation media is prepared by adding K$_2$HPO$_4$- 2g, CaCl$_2$ – 0.3g, MgSO$_4$-7H$_2$O– 1g, NH$_4$NO$_3$- 2g, FeSO$_4$.7H$_2$O- 5mg, MnSO$_4$.4H$_2$O- 1.6mg, ZnSO$_4$.7H$_2$O- 3.45mg, CoCl$_2$.6H$_2$O- 2mg, Carboxy methyl cellulose salt- 5g and Distilled water- 1000ml. 100ml of this media is transferred to 8 clean and dried Erlenmeyer conical flask of 250ml capacity, 4 for each isolate, and their mouths were plugged with cotton wools. All flasks were sterilized by moist heat at 15lb for 15min and cooled to use for inoculation.

ENZYME PRODUCTION
5ml sterilized water poured to slant of selected isolates and scrapped via nichrome wire loop. 1ml spore suspension was transferred aseptically to each flask and incubated at 30ºC for 2, 4, 6 and 8 days under stationary condition.

ENZYME EXTRACTION
Culture filtrate having crude cellulase was harvested by filtration using Whatman No. 1 filter paper and stored in refrigerator at 4ºC.

ENZYME ACTIVITY
Enzyme activity was assayed using carboxy methyl cellulose (CMC) as substrate. The assay was performed on the basis of red brown color produced by dinitro salicylic acid (DNS) reagent. In this assay, 1ml of 2% CMC solution in 0.05M sodium citrate buffer of pH 5.0 was taken in glass test tube and added 0.5ml culture filtrate then mixed by using cyclomixer. All test tubes with reaction mixer were incubated in water bath at 30ºC for 15min then added 2ml DNS reagent (DNS- 10g, Phenol- 2g, Sodium succinate- 0.5g, sodium hydroxide- 10g and Distilled water- 1000ml) to stopped enzyme - substrate reaction. All test tubes were incubated for 20 min in boiling water bath to developed red brown color. All test tubes were takeout from water bath and cooled then added 1ml of 1% Rochella salt (Sodium potassium tartrate- 10g and Distilled water- 1000ml). All test tubes were left at room temperature for 20min to stabilized red brown color. Color density was recorded by spectrophotometer at 550nm using spectrophotometer and compared with standard curve of glucose (1mg/ml). Enzyme activity was determined in international unit (IU) as an amount of enzyme that produces 1µM glucose/min.
RESULTS AND DISCUSSION

IDENTIFICATION OF THE FUNGI

The colony morphology and the microscopic observation by lacto phenol and cotton blue stain confirmed the organisms to be *Aspergillus* and *Rhizopus* spp.

SCREENING MEDIA COMPOSITION

The media used for screening of cellulase producing fungi is as follows:

**Table 1- Screening media composition.**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of the Chemical</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaNO₃</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>K₂HPO₄</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>MgSO₄.7H₂O</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>KCl</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>Carboxymethylcellulose sodium salt</td>
<td>2.0</td>
</tr>
<tr>
<td>6</td>
<td>Peptone</td>
<td>0.2</td>
</tr>
<tr>
<td>7</td>
<td>Agar agar</td>
<td>17.0</td>
</tr>
<tr>
<td>8</td>
<td>Distilled water</td>
<td>1000</td>
</tr>
</tbody>
</table>

FERMENTATION MEDIA COMPOSITION

The media used for cellulase production by submerge fermentation technology is as follows:

**Table 2- Production Media Composition.**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of the Chemical</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NH₄NO₃</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>K₂HPO₄</td>
<td>2.0</td>
</tr>
<tr>
<td>3</td>
<td>MgSO₄.7H₂O</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>FeSO₄.7H₂O</td>
<td>0.005</td>
</tr>
<tr>
<td>5</td>
<td>Carboxymethylcellulose sodium salt</td>
<td>5.0</td>
</tr>
<tr>
<td>6</td>
<td>CaCl₂</td>
<td>0.3</td>
</tr>
<tr>
<td>7</td>
<td>ZnSO₄.7H₂O</td>
<td>0.00345</td>
</tr>
<tr>
<td>8</td>
<td>CoCl₂.6H₂O</td>
<td>0.002</td>
</tr>
<tr>
<td>9</td>
<td>MnSO₄.4H₂O</td>
<td>0.0016</td>
</tr>
<tr>
<td>10</td>
<td>Distilled water</td>
<td>1000</td>
</tr>
</tbody>
</table>
ENZYME ACTIVITY

Culture filtrates of *Rhizopus* spp

Table 3- Enzyme estimation from culture filtrates of *Rhizopus* spp.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Incubation days</th>
<th>Enzyme activity (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>0.106</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0.346</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>0.446</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>0.503</td>
</tr>
</tbody>
</table>

Fig. 1- Enzyme estimation from culture filtrates of *Rhizopus* spp.

Table-3 show that amount of cellulose was increased with incubation time in submerge culture condition by *Rhizopus* spp. Further fig.-1 reveals that maximum amount of cellulose was increased after 8 days incubation time as 0.5IU/ml.

Culture filtrates of *Aspergillus* spp

Table 4- Enzyme estimation from culture filtrates of *Aspergillus* spp.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Incubation days</th>
<th>Enzyme activity (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>0.130</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0.411</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>0.502</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>0.571</td>
</tr>
</tbody>
</table>
Fig. 2- Enzyme estimation from culture filtrates of *Aspergillus* spp.

Results of table-4 and fig.-2 reveal that amount of cellulose was increased with incubation time in submerge culture condition. 0.13 and 0.571 IU/ml cellulase was available in culture filter of *Aspergillus* after 2 and 8 days incubation time respectively.

Initially CMC was slowly hydrolyzed by both fungi, as incubation time increase then concentration of enzyme was also increased. After 4 days, fungal cell undergoes stationary condition then amount of enzyme was increased slowly till 8 days incubation time. As compared to both fungi *Aspergillus* produced more cellulase than *Rhizopus*. These observations are also supported by Jahangeer *et al.* [6], Pothiraj [12], Abd-Elazher and Fadel [14] and Gilna and Khaleel [7] that fungal cellulase produced in both surface and submerge fermentation condition are hydrolyzed cellulosic material and produces reducing sugar as glucose units. This glucose have been used for many purposes i.e. food and beverages.

**CONCLUSION**

Cellulose is an enzyme that has cellulolytic ability and produce sugar oligomers. Much of the study is done in surface or solid fermentation with fungal microorganisms. In this study we used two different fungal species for cellulase production in submerge fermentation during varying incubation time. The results reveal that maximum cellulase production is achieved by *Aspergillus* and also proves that *Aspergillus* exhibits best growth in submerge fermentation condition than *Rhizopus*. 
REFERENCES


