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Cellulase Production by Liquid State Fermentation of Bacillus Species Isolated from Woody Forest Soil

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Abstract: *A Bacillus species identified as Bacillus cereus was isolated and characterized by enriching the basal culture medium with filter paper as substrate for cellulose degradation, the culture was found to possess cellulolytic activity, as demonstrated by degradation of carboxy methyl cellulose on cellulose congo red agar media. Also the Cellulose-degrading potential of the isolate was qualitatively estimated by calculating hydrolysis capacity (HC), that is, the ratio of diameter of clearing zone and colony. The enzyme assays for two enzymes, filter paper cellulase (FPC), and cellulase (endoglucanase), were examined by methods recommended by the International Union of Pure and Applied Chemistry (IUPAC). Enzyme assay for cellulase activity on filter paper was found to be 0.33 IU/mL, while for endoglucanase assay maximum activity was determined to be 0.64 IU/mL. The temperature was maintained at 37ºC and pH at 7±0.2, as these were the optimal conditions of cellulose production. Enzyme production was slightly affected at temperatures <30º and> 40º C but was drastically affected at temperatures < 20º and > 45º C.*

Keywords: *Bacillus cereus, cellulose, endoglucanase, FPCase*

1. INTRODUCTION

Cellulases (3.2.1.4) have a wide range of industrial applications such as textile, laundry, pulp and paper, fruit juice extraction, and animal feed additives as well as in bioethanol production [1]. Majority of studies on cellulase production have focused on fungi, with relatively lesser emphasis on bacterial sources [1]. Cellulase can be produced by fungi, bacteria or actinomycetes but bacteria which have a high growth rate as compared to fungi have good potential to be used in cellulase production. Bacillus sp. continues to be dominant bacterial workhorses due to the capacity of some selected species to produce and secrete large quantities of extracellular enzymes [2-7]. The purpose of the present study was to isolate cellulose degrading bacteria from forest soil where the organisms compete for their carbon source and degrade easily available cellulose into utilizable form of sugars. The qualitative and quantitative assessment of cellulolytic potential of isolated bacteria was carried out.

2. MATERIALS AND METHODS

The organism used for the study is a species of *Bacillus cereus*

2.1. Sample Collection:

Soil was collected for the isolation of cellulose degrading bacteria from different region of woody forest area of *Pachmarhi*, M.P.

2.2. Isolation of Bacteria:

Soil inoculated in Basal Salt Media with filter paper

↓

Incubated for 7 days at 37°C at 100rpm

↓

Transferred with wireloop on cellulose agar media

↓

Colonies showing discoloration of Congo red selected

↓

Identified as Bacillus

1 gram of soil was inoculated in a basal salt media (NaNO3 2.5g; KH2PO42g; MgSO4 0.2g; NaCl 0.2g; CaCl2·6H2O 0.1g in a liter) containing filter paper (What man filter paper no. 1 of 1 x 6 cm) for the isolation of cellulolytic bacteria. These cultures were incubated for 7 days in a shaker incubator at 37°C at 100rpm. Bacterial colonies capable of utilizing cellulose as sole source of carbon were isolated on cellulose agar media composed of KH2PO4 0.5g MgSO4 0.25g cellulose 2.0g agar 15g gelatin 2g and distilled water lL and at pH 6.8–7.2.

Confirmation of cellulose-degrading ability of bacterial isolates was performed by streaking on the cellulose Congo-Red agar media with the following composition: KH2PO4 0.5g, MgSO4 0.25g, cellulose 2g, agar 15g, Congo-Red 0.2g, and gelatin 2g; distilled water 1L and at pH 6.8–7.2. The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria. Colonies showing discoloration of Congo-Red were taken as positive cellulose-degrading bacterial colonies [8], and only these were taken for further study. Cellulose-degrading potential of the positive isolates was also qualitatively estimated by calculating hydrolysis capacity (HC), that is, the ratio of diameter of clearing zone and colony.

All the experiments were performed in triplicate. The culture broth after 36h of incubation was centrifuged at 10,000g for 10min at 4°C to separate the cells. The cell-free supernatant was analyzed for enzyme activity and protein concentration. The enzyme assays for two enzymes, filter paper cellulase (FPC), and cellulase (endoglucanase), were examined by methods recommended by the International Union of Pure and Applied Chemistry (IUPAC).

3. ENZYME PRODUCTION

The selected CDB isolates were cultured at 37°C at 150rpm in an enzyme production media composed of KH2PO4 0.5g, MgSO4 0.25g, and gelatin 2g, distilled water 1L and containing What man filter paper No.1 (1 × 6 cm strip, 0.05g per 20mL) and at pH 6.8–7.2. Liquid state fermentation process was started in 250 ml flask containing 100 ml of media with 1 ml of Broth culture after twelve days of incubation period was subjected to centrifugation at 5000rpm for 15min at 4°C. Supernatant was collected and stored as crude enzyme preparation at 4°C for further enzyme assays.

4. DETERMINATION OF ENZYME CONCENTRATION

The protein content of all isolated enzymes was determined by the Lowry's method using BSA as a standard. The concentration of protein was calculated by interpolation of standard graph prepared by using prism 6 software.

5. ASSAY OF ENZYME

5.1. Filter Paper Assay (FPA)

FPA is the most common total cellulase activity assay recommended by the International Union of Pure and Applied Chemistry (IUPAC). IUPAC recommends a filter paper activity (FPA) assay that differs from most enzyme assays based on soluble substrate for initial reaction rates. This assay is based on a fixed degree of conversion of substrate, i.e. a fixed amount (2 mg) of glucose (based on reducing sugars measured by the DNS assay) released from 50 mg of filter paper (i.e., both amorphous and crystalline fractions of the substrate are hydrolyzed) within a fixed time (i.e., 60 min). In part due to the solid heterogeneous substrate, reducing sugar yield during hydrolysis is not a linear function of the quantity of cellulase enzyme in the assay mixture. That is, twice the amount of enzyme does not yield two times the reducing sugar within equal time. Total cellulase activity is described in terms of "filter-paper units" (FPU) per milliliter of original (undiluted) enzyme solution. The strengths of this assay are that (1) the substrate is widely available and (2) the substrate is reasonably susceptible to cellulase activity. However, the FPA has long been recognized for its complexity and susceptibility to operator errors.

6. RESULTS AND DISCUSSIONS

The FPA of the original concentrated enzyme solution was calculated in terms of FPU/ml:

Where, 2 mg glucose = 2 mg/(0.18 mg/mmol) × 0.5 ml × 60 min = 0.37 mmol/min/ml

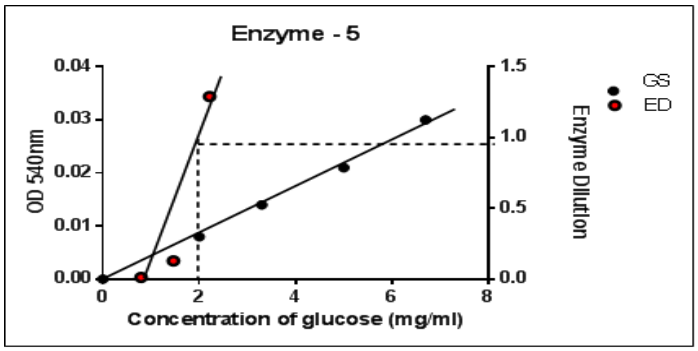

Figure 1. *FPCase of the enzyme sample from F5.*

Figure 1: Enzyme 5 is produced by isolate named F5. Here GS is Glucose Standards and ED is Enzyme Dilution. OD 540 refers to optical density at 540 nm.

(Graphs prepared by prism 6)

Table 1. *EDR for FPCase activity from Figure 1*

CDB Isolates	F5
FPCase EDR	1.13

6.1. Endoglucanase Assay Using CMC/DNS (CMCase activity)

The IUPAC-recommended endoglucanase (CMCase) assay is a fixed conversion method, which requires 0.5 mg of absolute glucose released under the reaction condition [9]. The reducing end concentration is measured by the DNS method. The enzyme dilution series was prepared of which at least two dilutions must be made of each enzyme sample, with one dilution releasing slightly more than 0.5 mg of glucose and one slightly less than 0.5 mg of glucose. The following steps were followed [10].

1. Add 0.5 ml of the dilute enzyme solutions (DESs) into test tubes with a volume of at least 25 ml.
2. Equilibrate the enzyme solution and substrate solution at 50°C.
3. Add 0.5 ml of the CMC solution to the test tubes and mix well.
4. Incubate at 50°C for 30 min.
5. Add 3.0 ml of DNS solution and mix well.
6. Boil for exactly 5.0 min in vigorously boiling water.
7. Place the tubes in an ice-cooled water bath to quench the reaction.
8. Add 20 ml of distilled water and seal with parafilm or by a similar method. Mix by inverting the tubes several times.
9. Read the absorbance at 540 nm based on the substrate blank.
10. Prepare the substrate blank (0.5 ml of CMC solution + 0.5 ml of citrate buffer) & enzyme blanks (0.5 ml of CMC solution + 0.5 ml of dilute enzyme solutions). Treat substrate and enzyme blanks identically as the experimental tubes.
11. Prepare the glucose standards:

    GS1 – 0.125 ml of 2 mg/ml glucose + 0.875 ml of buffer.

    GS2 – 0.250 ml of 2 mg/ml glucose + 0.750 ml of buffer.

    GS3 – 0.330 ml of 2 mg/ml glucose + 0.670 ml of buffer.

    GS4 – 0.500 ml of 2 mg/ml glucose + 0.500 ml of buffer.

12. Calculate the glucose released by the enzyme solutions with deduction of the enzyme blank absorbance based on the glucose standard curve.

13. Draw the relationship between the real glucose concentrations and their respective EDRs.
14. Link the points less than 0.5 mg and greater than 0.5 mg by a line, and identify the EDR by using the point for 0.5 mg glucose.

Calculate the CMCase activity of the original concentrated enzyme solution in terms of IU/ml:

$$\text{CMCase} = \frac{0.185}{\text{EDR}}$$

Where, 5 mg glucose = 5 mg/(1.8 mg/mmol) × 0.5 ml × 30 min = 0.185 mmol/min/ml. The EDR values obtained from figure 1 is shown in table 1.

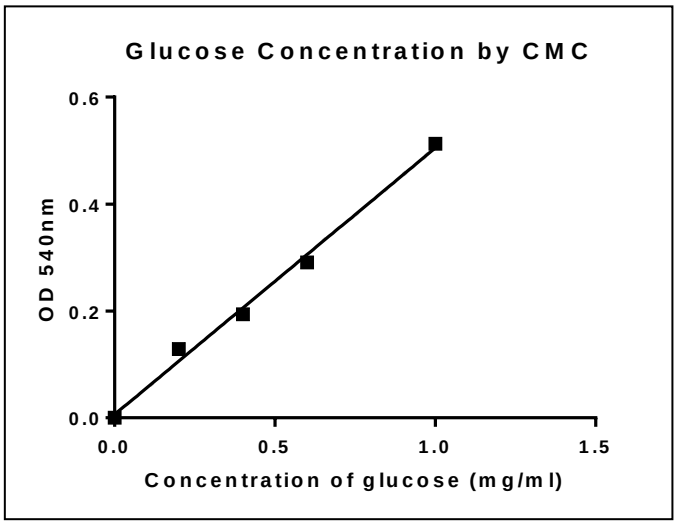

Figure 2. *Standard curve of glucose and EDR calculation for CMCase of each enzyme sample.*

Enzyme 5 is produced by F5 CDB isolate. Here GS is Glucose Standards and ED is Enzyme Dilution. (Graphs prepared by prism 6).

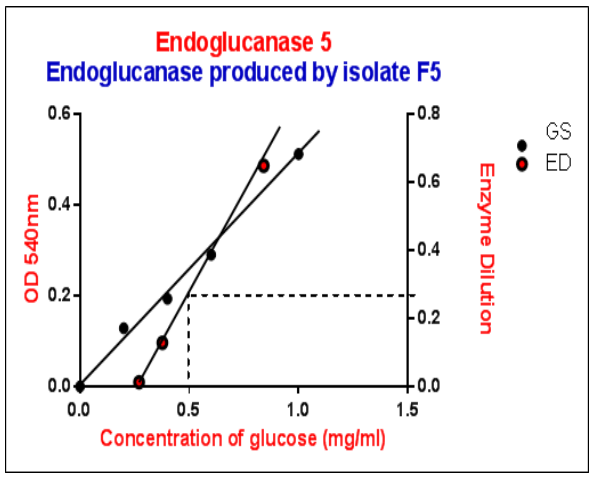

Figure 3. *Standard curve of glucose and EDR calculation for CMCase of each enzyme sample*.

Table 2. *EDR for CMCase activity from Figure 3*

CDB Isolates	F5
CMCase EDR	0.29

Table 3 shows the assessment of the bacterial isolate for cellulose decomposition via measurement of clear zone around the colony and calculation of hydrolytic value.

Table 3. *Maximum clearing zone and hydrolytic capacity (HC) value of CDB on cellulose Congo red agar media*

Isolate	Average zone diameter (mm)	colony diameter (mm)	HC
F5	40	40	4.44

6.2. Protein Determination

Following results were obtained for protein determination:

The interpolated concentration values were obtained from the curve shown in figure 6 using prism 6 software trial versions. The concentration of protein was found to be 1.29 mg/ml.

Table 4. *Protein concentration of the enzyme.*

Cellulase	protein (mg/ml)
Enzyme 5	1.29

6.3. Assessment of Cellulolytic Potential

Table 5. *Enzyme Dilution Rates for FPCase & CMCase from Figure 1 and 3*

CDB Isolates	FPCase EDR	FPCase Activity (IU/ml)	CMCase EDR	CMCase Activity (IU/ml)
F5	1.13	0.33	0.29	0.64

7. CONCLUSION

The isolate *B.cereus* possesses good amount of FPCase and CMCase activity. The estimation was done using the standard conditions. The optimization of physical and chemical conditions may help improve the enzyme activity.

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