Production and optimization of cellulase from *Fusarium oxysporum* by submerged fermentation

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*Fusarium oxysporum*, isolated from infected tomato plant parts, produced maximum cellulase at optimum parameters (pH, 6.0; temp., 50°C; and incubation period, 12 d) in cellulase enzyme production broth having 1% CMC (carboxy methyl cellulose) as a cellulose substrate. Activities of purified cellulases (mol wt, 24, 29 and 45 kDa) were stimulated by concentrations (0-70 mM) of Na\(^+\) and Mg\(^{++}\), while EDTA inhibited enzyme activity at all concentrations.

**Keywords:** Cellulose, CMC, Fermentation, *Fusarium oxysporum*, Glucosidase

**Introduction**

Cellulose is most common carbohydrate on earth\(^1\). Utilization of cellulose as a nutrient source requires enzymes that cleave β-1, 4- glycosidic bonds between constituent sugars\(^2\). Cellulase is associated with pathogenicity of a number of microorganisms\(^3\). *Fusarium oxysporum*, a cosmopolitan soil borne filamentous fungus, is an anamorphic species that includes numerous filamentous plant pathogenic strains causing wilt disease of a broad range of agricultural and ornamental host plant species. It produces several enzymes that act upon pectic and cellulose components of cell walls\(^4\). This study presents production and optimization of cellulose enzyme from *F. oxysporum* by submerged fermentation.

**Experimental Section**

Sample Collection and Assessment of Cellulase Activity

Infected parts (leaf, stem) of diseased tomato plant were collected and aseptically transferred to laboratory for further isolation of fungal pathogen. Fungal colonies were isolated in potato dextrose agar (PDA) plates. Cellulose degradation potential was assessed of selected strains by growth and zone formation in carboxy methyl cellulose (CMC) agar medium.

Liquid State Fermentation

Cellulase enzyme production medium and inoculated spore suspensions of *F. oxysporum* were prepared. Broth cultures were incubated on a rotatory shaker (150 rpm) at 30°C. Fermented samples were withdrawn at two days (2, 4, 6, 8, 10, 12 days) interval. Reducing sugar was estimated by DNS (Dinitro Salicylic acid) method\(^5\) and protein content by reported method\(^6\).

Assay of Cellulase Enzyme

Activity of cellulase was assayed by reported method\(^7\). Fermented sample was centrifuged at 3000 rpm for 10 min. Supernatant was used as enzyme source. For CMCase activity, suitably diluted enzyme solution (1 ml diluted equally with 0.2 M acetate buffer, pH 4.8) was incubated with 1 ml CMC (10 mg/ml) in 0.2 M acetate buffer at 60°C for 30 min. For FPase activity, suitably diluted enzyme solution (1 ml diluted equally with 0.2 M acetate buffer) was incubated with 50 mg filter paper (What man No.1) in 1 ml of 0.2 M acetate buffer (pH 4.8) for 1 h at 60°C. For β-glucosidase activity, suitably diluted enzyme solution (1 ml diluted with 0.2 M acetate buffer) was incubated with 1 ml of salicin (10 mg/ml) solution in 0.2 M acetate buffer (pH 4.8) at 60°C for 30 min. Amount of reducing sugars released in CMCase, FPase, β-glucosidase assay after incubation was estimated by DNS method\(^5\). Cellulase was estimated\(^8\) and its activity was expressed in International Unit (IU).

Effect of Cations and EDTA on Cellulase Activity

Effect of cations on cellulase activity was determined by using two cations (Na\(^+\) and Mg\(^{++}\); conc., 0, 10, 20, 30, 40, 50, 60 and 70 mM). Substrate – cation mixture was incubated at room temperature (RT) for 1 h before it was...
used in enzyme assay. Effect of ethylene diamine tetraacetic acid (EDTA) at various molar concentrations (0, 10, 20, 30, 40, 50, 60 and 70 mM) on activity of cellulase was determined. Substrate – chemical compound mixture was incubated at RT for 1 h before it was used in enzyme assay.

**Optimization of Culture Conditions**

Optimum culture conditions [pH (4, 5, 6, 7, 8 and 9), temperature (30, 40, 50, 60, 70 and 80°C), incubation period, carbon (glucose, lactose, sucrose and cellulose; 1% w/v) and nitrogen (peptone, yeast extract, urea, diammonium sulphate; 1% w/v) source requirements] were determined for maximum growth of *F. oxysporum* and cellulase activity were recorded.

**Crude Protein Extraction by Ammonium sulphate Precipitation**

Ammonium sulphate (60%) was added to filtrate slowly with continuous stirring at low temperature (in an ice bath/beaker) for 5-10 min and left overnight in refrigerator. Mixture (salt+filtrate) was centrifuged at 12000 rpm for 20 min at 4-6°C, then pellet was collected and dissolved in 0.2 M sodium phosphate buffer (pH 7.0) using two volumes of buffers. During collection of enzymes, tubes and funnel were washed with same buffer and collected; process was repeated thrice. Volume should not be 20% of total volume of filtrate. Enzyme salt solutions were dialysed with same buffer, which was changed 3-5 times for removing ammonium salt.

**Double-Layer Plate Assay**

To detect crude enzyme activity, a bottom layer [15 ml of 0.7% (w/v) agarose and 50 mM potassium phosphate-citric acid buffer (pH 5.2)] was overlaid with 5 ml of CMC (0.2%; w/v) and agarose (0.5%; w/v). Plates were inoculated with protein extract (40 µl) at 30°C for 24 h. To detect CMCase activity, plates were flooded with congored solution (1%; w/v) for 30 min and then rinsed several times with 1 M NaCl. This procedure revealed distinct hydrolysis regions.

**Purification by Ion- Exchange Chromatography on DEAE - Sephadex A - 50**

DEAE sephadex A – 50 column was packed into a vertically mounted column (1.5 cm x 40 cm) at a flow rate of 30 ml/h. Column was equilibrated with 3 bed volumes of 0.02 M sodium phosphate buffer, pH 7.0 containing 1 mM 2-mercaptoethanol. Enzyme concentrate obtained from ammonium sulphate precipitation was redissolved in minimal amount of buffer and dialysed for 24 h against 0.02 M sodium phosphate buffer, pH 7.0 containing 1 mM EDTA and 1 mM 2-mercaptoethanol with two changes of buffer per hour. Undissolved precipitate was removed by centrifugation and clear supernatant was layered on prepared column. Column was washed to remove all unbound proteins and a linear gradient of 0-0.5 M NaCl in 0.02 M sodium phosphate buffer (pH 7.0) was used to elute any bound proteins. Fractions containing cellulase activities were pooled together and precipitated with ammonium sulphate. Precipitate was collected by centrifugation at 4000 g in a cold centrifuge at 4°C for 30 min, redissolved in 2.5 ml of 0.02 M sodium phosphate buffer (pH 7.0) and dialysed against buffer (pH 7.0) for 6 h.

**Molecular Mass Determination**

Molecular weight of cellulase was determined by SDS-PAGE using selectable markers.

**Results and Discussion**

**Assessment of Cellulase Activity in CMC Agar Medium**

Selection of over producing cellulase strains of *F. oxysporum* were based on diameter of clearing zone surrounding small well in plate screening medium. Size of clearing zone diameter for selected strain was recorded for different incubation period as follows: 2 days, 12 mm; 4 days, 24 mm; 6 days, 32 mm; and 8 days, 36 mm. Enzyme activity of *F. oxysporum* was found as follows: CMCase, 1.92 U/ml; FPase, 1.34 U/ml; and ß-glucosidase, 1.78 U/ml.

**Cellulose**

Cellulose degradation was assessed on 8th day of fermentation by selected fungal strains. *F. oxysporum* utilized 32.68% of cellulose on 8th day of incubation (Fig. 1a). Similar results have been reported with studies on cellulosic substrates.

**Reducing Sugar**

Initial content of free reducing sugar in cellulase production broth was found to be 2.38 mg/ml. A gradual and steady increase in reducing sugar content was observed in cellulase production broth supplement with CMC. A tremendous (1.28 fold) increase in content of reducing sugar was observed during 8th day of fermentation period by *F. oxysporum* (Fig. 1b). Amount of reducing sugar increased with time of incubation with presence of enzyme when cellulose rich agro waste supplemented as a substrate.
Fig. 1—Liquid state fermentation media inoculated with *F. oxysporum* and supplemented with CMC: a) Cellulose utilization; b) Reducing sugar content; and c) Protein content.
Protein
Initial protein content of cellulase enzyme production broth was 0.35 mg/ml. Protein content gradually increased till 8th day of fermentation. *F. oxysporum* could produce 0.71 fold increase in protein content in same period. After incubation, protein content declined (Fig. 1c).

Enzyme Assay
Fermentation time (8 days) had optimum effect of *F. oxysporum* on enzyme production as follows: CMCase activity, 1.92 ± 0.005; filter paper degradation activity, 1.34 ± 0.003; and β-glucosidase activity, 1.78 ± 0.005 (Table 1).

Effect of pH, Temperature, C & N Sources on Cellulase Enzyme Production
At pH 6 and 7, selected fungal strains of *F. oxysporum* showed heavy growth and higher cellulase activity
Maximum growth and enzyme production were recorded at 50°C liquid state fermentation (Fig. 2b). At 50°C, confluent growth and better enzyme activity was noticed. β-Glucosidase activity (0.769 U/ml), observed in *Fusarium oxysporum* when lactose supplemented as a sole carbon source, was higher than FPase (0.654 U/ml) and CMCase (0.542 U/ml) activity (Fig. 2c). Cellulase biosynthesis required residual inducer in culture medium for 2 days, when cellulose and lactose were used as growth substrate. In *Fusarium oxysporum*, enzyme activity got influenced when urea supplemented as a nitrogen sole source.

**Effect of Cations and EDTA on Cellulase Enzyme Production**

CMCase, FPase and β-glucosidases activity were inhibited with increasing concentration of EDTA (Fig. 3a). Cellulase activity was observed with decreasing concentration of Mg²⁺ ions by *Fusarium oxysporum* (Fig. 3b). Maximum cellulase activity was observed without addition of sodium for selected fungal strains. Drastic reduction of enzyme activity was observed with increasing concentration of sodium ions (Fig. 3c).

**Extraction of Crude Cellulase Enzyme**

Crude cellulase enzyme of *Fusarium oxysporum*, recovered following 60% saturation of culture supernatant with ammonium sulphate, showed an increase of specific activity.

**Molecular Weight**

Molecular weight of partially purified cellulase seemed to be 24, 29 and 45 kDa in *Fusarium oxysporum*. Purified enzymes of *Fusarium oxysporum* (three distinct bands) have been observed with the molecular weight of 24, 29 and 45 kDa.

**Conclusions**

Production and optimization of cellulase has been found suitable for *Fusarium oxysporum* and optimization of pH, temperature, incubation time, carbon and nitrogen sources, EDTA and cations are limiting factors for maximum cellulase enzyme production as well as enzyme activity. Therefore, *Fusarium oxysporum* is a potential fungal strain for production of cellulase enzyme.

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**References**