Screening and Identification of Cellulase-Producing Strain of Fusarium Oxysporum

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Abstract

Five strains were screened out from 120 strains of Fusarium Oxysporum Schl. isolated from different regions. Based on ITS analyses, the strains were determined as Fusarium Oxysporum Schl. The strain named as H57-1 produce the highest cellulase, which activity reach at 1.43 IU/mL. The most suitable reactive pH of the CMCase was 4.5–5.5, and the most suitable reactive temperature is 60–65\textdegree C. Its activity is stable at pH 5.3–9.5 and 4–50\textdegree C.

1. Introduction

Cellulase is made of three components: endoglucanase exoglucanase, beta-glycosidase. The nature cellulose was completely degradation to glucose by cellulase\textsuperscript{[1-3]}. The cellulase is widely used in food, brewing, textile, papermaking, feed, oil etc. Cellulase is mainly produced by the microbes, especially the fungus, such as Aspergillus and Penicillium. Fusarium oxysporum is also a fungus which can degradate cellulose. But the study on the cellulase produced by Fusarium oxysporum is very little. We have isolated many strains from the flax. The present study was aimed at screening a strain which has high production of endoglucanas.
2. Material and methods

2.1 Microorganism and medium

Fusarium oxysporum, a new fungus isolate from the flax plant infected wilt, was used. The potato-dextrose-broth (PDB) was used as the inoculum medium. The fermentation medium contained (g/L): bran 30, CMC-Na 5, peptone 10, KH$_2$PO$_4$ 1, MgSO$_4$ 0.2, (NH$_4$)$_2$SO$_4$ 3. pH was adjusted to 6.0 with 0.1N NaOH or HCl. The organism was maintained on potato- dextrose-agar (PDA) slants at 4°C.

2.2 Fermentation

The organism was incubated on PDA plate at 28°C for 4 days. 2cm$^2$ mycelium was incubated in the inoculum medium grown under agitation (150r/min) in a shaker at 28°C for 24h. The batch fermentations were carried out in a 250ml flask under agitation (150r/min) at 28°C for 48h. The medium volume was 30ml. The inoculum was 1% (v/v).

2.3 Strains screening

The fermentation broth was added to the hole on the sodium carboxymethylcellulose (CMC-Na) plate (CMC-Na plate contained (g/L): CMC-Na 2, agar 18, pH was adjusted to 6.0 with 0.1N NaOH or HCl.) Each hole added 400 microliter broth. After 16h, 0.2g/L congo red dye liquor was added for half an hour$^4$. Abandoned the dye liquor, 40g/L NaCl was added for half an hour. The vernier gauges was used to determine the diameter of the hydrolysis circle. The strains, which formed large hydrolysis circle, was kept.

2.4 Enzyme assay

After cultivate for 48h, the broth was centrifuged at 4500r/min for 20min. The supernatant obtained was used as the crude enzyme extract. Cellulase activity was measured by the DNS (3,5-dinitrosalicylic acid) method$^5$, through the determination of the amount of reducing sugars liberated from 2%(w/v) CMC-Na solubilized in 50mM citrate buffer, pH4.8. This mixture was incubated for 30min at 50°C and the reaction was stopped by the addition of DNS solution. The treated samples were boiled for 10min, cooled in water for color stabilization, and the optical density was measured at 540nm. The cellulase activity was determined by using a calibration curve for glucose. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of glucose per minute and was expressed as units per milliliter(U/mL).

2.5 Sequencing and assay of ITS rDNA [6~8]

The genomic DNA was extracted according to Fernando, W. G. D.$^9$. PCR assays were carried out
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using the primers below: ITS1: 5'-TCC GTA GGT GAA CCT GCG G-3'; ITS4: 5'-TCC TCC GCT TAT TGA TAT GC-3'. Amplification reactions were performed in 50 μl containing: 5 μl 10×reaction buffer, 4 μl of 25 mM MgCl₂, 4 μl of 10 mM dNTPs mix, 1 μl of 10 μM each primer, 1μl Taq DNA polymerases (TaKaRa, Japan) and 2 μl DNA template. All PCR reactions were performed in TGradient Thermal Cycler (Biometra, Germany) using an initial 5 min denaturation at 94°C followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, 90 s extension at 72°C, followed by final extension of 5 min at 72°C. Amplified products (15μl) were loaded on a 1% (w/v) agarose gel with TAE buffer. Amplification product of ITS region was extracted from agarose gel and the 500 bp band was purified by using TaKaRa gel extraction kit (TaKaRa, Japan). The PCR product was cloned into pMD-19T easy vector (TaKaRa, Japan) according to the manufacturer’s instructions and ligation mix was used to transform DH5α competent cells. Screening of transformed colonies was achieved by culture on LB medium supplemented with antibiotic (100 mg Ampicillin/L). Plasmid DNA extraction was performed using Plasmid Mini kit (Qiagen, Germany). Recombinant DNA plasmid was sequenced by INVITROGEN Co. Ltd. (Shanghai, China). DNA sequences were analyzed and aligned using BLAST.

2.6 Effect of temperature on enzyme activity and stability

The optimum temperature of the crude enzyme for hydrolysis of CMC-Na in 50mM citrate buffer (pH4.8) was determined by incubating the mixture of the crude enzyme and 2% (w/v) CMC-Na for 30min at different temperatures ranging from 25 to 85°C. The reaction was stopped by the addition of DNS solution. Heat stability studies of the crude enzyme were performed by incubating the crude enzyme in 50mM citrate buffer (pH4.8) at temperatures ranging from 4 to 80 °C for 60min. The residual activity of each sample for hydrolysis of CMC-Na was then quantified at 50 °C for 30min with the DNS method.

2.7 Effect of pH on enzyme activity and stability

The optimum pH of the crude enzyme was determined by incubating the mixture of the crude enzyme and 2% (w/v) CMC-Na in the presence of appropriate buffers: 50mM citrate buffer (pH2.8–6.0), 50mM sodium phosphate (pH 6.0–8.0), 50mM Tris–HCl (pH 8.0 and 9.0) and 50mM glycine–NaOH (pH 9.0–10.3). The reaction mixtures in various pH buffers were incubated for 30min at 50°C and the cellulase activity was assayed by DNS method. The pH stability studies were performed by incubating the crude enzyme for 60min at 50 °C and at a pH ranging from 2.8 to 10.3. The residual activity of each sample for hydrolysis of CMC-Na was then quantified at 50 °C for 30 min with the DNS method.

3. Results and discussion

3.1 Strains screening

Twenty strains, which formed larger hydrolysis circle (Fig. 1) on CMC-Na plate, were chosen to fermentation in flask. Five strains produced more enzyme yield(Table 1). Especially, the enzyme yield
produced by strain H57-1 is 1.43U/ml.

3.2 Sequencing and assay of ITS rDNA

The five strains screened were analyzed and identified microbiologically under the microscope as *Fusarium* sp. (data not shown). The five strains was further characterized at molecular level by PCR amplification using ITS1/ITS4 primers as *Fusarium oxysporum* (Fig. 2).

![Fig. 1. Hydrolysis circle on CMC-Na plate](image_url)

![Fig. 2. Amplification of a 500 bp fragment of the ITS from Fusarium oxysporum](image_url)

<table>
<thead>
<tr>
<th>No.</th>
<th>Diameter (cm)</th>
<th>Yield(U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H57-1</td>
<td>2.6</td>
<td>1.43</td>
</tr>
<tr>
<td>H38-1</td>
<td>2.5</td>
<td>1.09</td>
</tr>
<tr>
<td>X39-2</td>
<td>2.5</td>
<td>1.10</td>
</tr>
<tr>
<td>H16-1</td>
<td>2.4</td>
<td>1.19</td>
</tr>
<tr>
<td>X14-6</td>
<td>2.6</td>
<td>1.09</td>
</tr>
</tbody>
</table>

Fig. 2. Amplification of a 500 bp fragment of the ITS from Fusarium oxysporum. Lane 1: strain H57-1. Lane 2: strain H38-1. Lane 3: strain X39-2. Lane 4: strain H16-1. Lane 5: strain X14-6. M: 250 bp DNA Ladder Marker. PCR products were separated on a 1% agarose gel and visualized with ethidium bromide.

3.3 Effect of temperature on enzyme activity and stability

The effect of temperature on the CMCase activity of the crude cellulase was determined at various temperatures ranging from 25 to 85°C at pH4.8 shown in Fig.3. After 30min of incubation, the residual activity in aliquots withdrawn at various times was assayed at 50°C for 30min under the standard
conditions. Relative activities for CMC-Na at 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 and 85°C were 24.3, 33.6, 42.5, 60.6, 64.5, 76.8, 86.1, 100, 98.1, 75.7, 69.1, 32.1 and 15.06, respectively. The crude cellulase was optimally most active at 60°C at pH 4.8.

The thermal stability of the crude cellulase was also determined at various temperatures ranging from 4 to 80°C at pH 4.8 shown in Fig. 4. More than 90% of the original CMCase activity of the crude cellulase was maintained at temperatures ranging from 4 to 50°C after 1h incubation at pH 4.8. About 60% of the original CMCase activity was maintained at broad temperatures ranging from 4 to 60°C whereas less than 40% of the original CMCase activity was observed at 70 and 80°C.

3.4 Effect of pH on enzyme activity and stability

The effect of the pH on the CMCase activity of the crude cellulase was examined at various pHs ranging from pH 2.8 to pH 10.3 as shown in Fig. 5. The optimal pH for the CMCase activity was pH 5.3.

The pH stability of the crude cellulase was also examined at various pHs ranging from pH 2.8 to pH 10.3, as shown in Fig. 6. More than 70% of the original CMCase activity of the crude cellulase was maintained at broad pHs ranging from pH 4.2 to pH 10.3 after 1h incubation at 50°C. The pH stability of the cellulase produced by *Fusarium oxysporum* H57-1 over a broad pH range seems to be a seldom
characteristic of many Bacillus endoglucanases.

![Graph showing effect of pH on activity of cellulase](image)

Fig. 5 Effect of pH on the activity of *Fusarium oxysporum* H57-1 cellulase. The enzyme was incubated at 50 °C with 2% (w/v) CMC at different pH buffer ranging from pH2.8 to pH10.3.

### 3. Conclusion

*Fusarium oxysporum* H57-1 was isolated from infected flax plant as a pathogen. A number of enzymes produced by *Fusarium oxysporum* have been reported. Strain H57-1 was screened because of the high yield of cellulase. In this study, a crude cellulase produced by *Fusarium oxysporum* H57-1 was characterized. The crude cellulase was optimally most active at 60°C, and about 60% of the original CMCase activity was maintained at broad temperatures ranging from 4 to 60 °C after 1h incubation. The optimal pH for the CMCase activity was pH5.3, and more than 70% of the original CMCase activity of the crude cellulase was maintained at broad pHs ranging from pH4.2 to pH10.3 after 1h incubation at 50°C.

![Graph showing pH stability of cellulase](image)

Fig. 6. The pH stability of *Fusarium oxysporum* H57-1 cellulase at different temperature. The enzyme was incubated at 50 °C with 2% (w/v) CMC at different pH ranging from pH2.8 to pH10.3.

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References


