Isolation and characterization of a novel lignocellulose decomposing fungal strain

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Received 26 May 2010; revised 23 November 2010

A strain F1 with high cellulase activity obtained from the deadwood stack was characterized as Ceriporia lacerate by examination of the general taxonomical characteristics and phylogenetic sequence analysis of rDNA ITS gene. The endoglucanase (EG) and filter paper cellulase (FPase) activities of the strain showed remarkable stability in the pH range of 4.0-7.0, and maintained about their maximal value of 76% and 50% after incubation at 70˚C for 6 h respectively. The strain grew particularly well with CMC-Na (1.0%) and yeast extract (0.4%) at 28˚C (pH 6.0) in flasks stirred at 150 × g for 6 days. Based on the thermostability and pH stability of cellulase, the strain appears to have potential in industrial applications and bioresource utilization.

Keywords: Cellulase, Ceriporia lacerate, Isolation, Identification, Lignocellulose

Biofuel is of rapidly growing interest in the field of energy security, diversity, and sustainability as well as for greenhouse gas mitigation1,2. Lignocelluloses can be converted to the products of commercial interest such as ethanol, lactic acid, single cell protein3,4. The production of cellulase is a major factor in the hydrolysis of cellulosic materials. The widely accepted mechanism for enzymatic cellulose hydrolysis involves synergistic actions by endoglucanase (EC 3.2.1.4), exoglucanase or celllobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21)5. Presently, the most widely studied cellulase, namely Trichoderma cellulase has shown several disadvantages. Attempts to use these enzymes in the bioconversion have not been successful due to several factors, such as: low enzymatic yields, low specific activities and end product inhibition of the enzyme6,7. Thus, isolation and selection of efficient cellulose-decomposing strains with complete enzyme system appear to be crucial.

In this paper, we report the isolation and identification of a cellulose-decomposing strain F1 with high enzyme activity from the deadwood stack. The thermostability and pH stability of the cellulase have been investigated and factors affecting cellulase production have also been optimized.

Materials and Methods

Culture medium

Filter paper medium and cellulose-Congo red medium were used as differential mediums8. The medium for submerged fermentation for cellulase production was similar to that reported previously9.

Isolation and screening of strains

Samples were collected from the composting of rice straw, deadwood stack, droppings of cattle and goat in the suburbs of Chongqing city (China) and diluted properly to inoculate into filter paper medium. The culture was kept under shaking at 30˚C until the filter paper turned into paste. This culture solution was inoculated on a cellulose-Congo red medium at 28˚C for incubation for 3 days. The colony with hydrolyzed circle was selected to screen the strain and was again inoculated on the isolation medium. The above-mentioned steps were repeated until the pure culture of the strain was obtained. The strains were inoculated into filter paper medium again to observe their degradation efficiency in 5 days.

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Abbreviations: EG, endoglucanase; FPase, filter paper cellulose.
Identification of the strain F1

Morphological and molecular analysis

Morphological analysis was carried out according to the Hand Book of Fungus Identification. For molecular analysis, the ITS rDNA was amplified using the universal primers set by PCR. The PCR products were cloned into pMD18-T vector (TaKaRa, Japan) and sequenced.

Preparation of crude enzyme

When fermentation was completed, the fermentation broth was leached by single-layer gauze, and then preserved at 4°C after centrifugation (7,500 × g, 4°C, 20 min) to remove the mycelium. The supernatant obtained was the crude cellulase.

Enzyme assay

The reducing sugar liberated in the reaction mixture was measured by the dinitrosalicylic acid (DNS) method. Filter paper cellulase (FPase) and endoglucanase (EG) were determined as reported earlier. The unit of enzyme activity was defined as 1 mL crude enzyme required to liberate 1 µmol of reducing sugars from the appropriate substrates per min under the assay conditions.

Cellulase production

Small scale experiments were carried out in 250 mL Erlenmeyer flasks with 50 mL fermentation medium, and cultured at 28–30°C, pH 5.5, 150 × g for 3 days.

Statistical analyses

Analyses of variance (ANOVA) were done with statistical analysis system (SAS, version 6.11). All experiments were performed in triplicate. The maximum difference among the three values was less than 5% of the mean. Fisher’s least significant difference (LSD) test was used to determine the significant differences among the means.

Results and Discussion

Screening of cellulose-decomposing strains

After screening of strains, 78 cellulose-decomposing strains were selected from the filter paper medium in the primary screening, and then 5 strains with high cellulase activity were selected from cellulose-congo red medium for secondary screening. The EG and FPase activity of strain F1 was higher than other strains. The diameter of hydroyzled circle and H/C (hydroyzed diameter/colony diameter), and degradation efficiency were also higher (Table 1), and the strain turned the filter paper into paste in less than 5 days. Thus, the strain F1 was selected for further studies.

Identification of cellulose-decomposing strain

Both morphological and molecular analyses were carried out to identify the strain. The characteristics of the strain F1 were similar with those of Ceriporia lacerate, and the rDNA ITS gene sequence showed 90% homology to C. lacerate (Fig. 1). Hence, the strain F1 was identified as C. lacerate F1 (GenBank Accession No. EU262985).

Effect of temperature on activity and stability of cellulase

The commercial cellulases are mainly extra-cellular enzymes produced by mesophilic or thermophilic fungi. Since the use of cellulose degrading enzymes produced by mesophilic or thermophilic fungi.

Table 1—Comparison of different criteria of various strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Diameter of hydrolyzed circle (cm)</th>
<th>H/C</th>
<th>EG (IU/mL)</th>
<th>FPase (IU/mL)</th>
<th>Degrading degree of filter paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>5.97 ± 0.15</td>
<td>2.44 ± 0.05</td>
<td>31.25 ± 0.06</td>
<td>25.92 ± 0.08</td>
<td>+++++++</td>
</tr>
<tr>
<td>F22</td>
<td>5.53 ± 0.15</td>
<td>1.03 ± 0.01</td>
<td>24.54 ± 0.03</td>
<td>9.72 ± 0.04</td>
<td>++++</td>
</tr>
<tr>
<td>F31</td>
<td>3.67 ± 0.16</td>
<td>1.08 ± 0.02</td>
<td>9.95 ± 0.04</td>
<td>2.89 ± 0.07</td>
<td>++</td>
</tr>
<tr>
<td>M57</td>
<td>4.60 ± 0.26</td>
<td>1.02 ± 0.01</td>
<td>17.36 ± 0.04</td>
<td>8.76 ± 0.01</td>
<td>+++</td>
</tr>
<tr>
<td>M63</td>
<td>4.92 ± 0.23</td>
<td>1.633 ± 0.03</td>
<td>8.33 ± 0.05</td>
<td>2.20 ± 0.02</td>
<td>+++</td>
</tr>
<tr>
<td>AS 3.3711</td>
<td>3.25 ± 0.13</td>
<td>0.99 ± 0.07</td>
<td>6.73 ± 0.12</td>
<td>2.18 ± 0.04</td>
<td>++</td>
</tr>
</tbody>
</table>

“+” indicates the degradation efficiency on filter paper.

H/C, hydrolyzed diameter/colony diameter, EG, endoglucanase FPase, filter paper cellulose.
enzymes is related to industrial processing and operating at high temperature, application of thermostable enzymes produced by mesophilic or thermophilic fungi appears to be advantageous.

In order to determine the effect of temperature on the cellulase activity of the strain F1, the standard procedure of the cellulase assay at different temperatures was performed. The optimum temperature of EG and FPase activities was 50°C (data not shown). The EG and FPase activities were maintained their maximal value of about 76% and 50% after incubation at 70°C for 6 h (Fig. 2). Moreover, the EG activity was retained over 60% of its maximal value after incubation even at 100°C for 6 h. Recently, it is reported that the EG activity produced by a thermoacidophilic fungus *Aspergillus terreus* M11 is maintained up to the maximum about only 65% after incubation at 70°C for 6 h. Therefore, the cellulase produced by the strain F1 may have great potential in the industry, due to its thermostability up to 100°C for 6 h. In particular, EG has been widely used in the textile and paper industry.

**Effect of pH on activity and stability of cellulose**

The pH stability analysis revealed that activity was stable over a broad pH (4.0-7.0) (Fig. 3). The residual activities of EG and FPase were maintained over 90% and 75% after incubation at pH 4.0 for 24 h, respectively. The optimum cellulase activity was found at pH 4.0 (not shown here), which was the similar to most cellulases (optimal pH range of 3.5–5.5) obtained from different microbial sources, indicating their potential in industrial applications.

**Effect of different carbon and nitrogen sources**

The carbon source used in cultivation is one of most important factors affecting the cost and yield of cellulose. Therefore, for reducing the cost of the enzyme, selection of a cheap and easily available substrate appears to be essential. We tested various lignocellulose carbon sources for their effect on cellulase production and found that the lignocellulosic materials were degraded easily by the strain F1, in spite of their complex structure (Fig. 4). Both FPase and EG activities reached the peak value, when only CMC-Na was used as carbon source. Thus, the strain F1 may find potential in degrading the lignocellulosic biomass, which is considered as a suitable substrate for cellulase production.

The effects of nitrogen sources on production of cellulase are found to vary, depending on the fungi.
and the compound tested\textsuperscript{21,22}. When different nitrogen sources were tested, the results showed that EG and FPase activities reached the peak value, when yeast extract was used as the sole nitrogen source (Fig. 5). Also, the enzyme activity was higher in the presence of utilizable organic nitrogen sources than inorganic nitrogen sources. These results were in accordance with previous some reports, though some studies reported optimal enzyme activities in the presence of inorganic nitrogen sources\textsuperscript{23,24}.

Effect of pH on the initial medium and incubation time of the cell growth

The maximal value of EG and FPase activities was obtained, when the initial pH values were 4.0 and 5.0, respectively (Fig. 6). Thus, the optimal pH of the strain F1 was the similar to the reported optimal culture pH (3.0-6.0) of fungi\textsuperscript{25}. Hence, it is suggested that the strain F1 could be used to degrade cellulosic materials and production of cellulase under acidic conditions. The cellulose producing ability of strain

F1 varied with the incubation time (Fig. 7). The maximal production was observed at 6\textsuperscript{th} day. When the strain incubated after 9 days, it hardly produced any cellulase. Time-course required reaching maximal value of EG and FPase activity may be affected by several factors, including the presence of different ratios of amorphous to crystalline cellulose\textsuperscript{26}.

Conclusion

In conclusion, the newly isolated strain F1 was identified as Ceriporia lacerate F1. The highest EG and FPase activity produced by the strain was 31.25 IU/ml and 25.92 IU/ml respectively. The cellulase was stable in pH range of 4.0-7.0 and at high temperatures (50-100°C). The residual activity was maintained its maximal value of 90% and 75% after incubation at pH 4.0 for 24 h and its maximal value of about 76% and 50% after incubation at 70°C for 6 h. The maximal production was observed at 6\textsuperscript{th} day (optimum initial medium pH 6). The strain grew particularly well with CMC-Na (1.0%) and yeast extract (0.4%), the preferred carbon and nitrogen sources respectively. The strain F1 may have potential in the degradation of straw, wheat bran, cotton, and other natural cellulos with the crystalline cellulose as the main part. However, since it is a white rot fungus, further work is required on the analysis of the crude xylanase and improvement of degradative activity of the strain F1.

Acknowledgements

The work was supported by the grants from Chongqing Nature Science Foundation Project of China (CSTC 2007BA1005) and State Nature Science Foundation Project of China (No. 30972002).
References