Preparation of Mutants Resistant to Catabolite Repression of Trichoderma reesei†

Mikio KAWAMORI, Yasushi MORIKAWA,* Yoriko SHINSHA, Kenichiro TAKAYAMA and Seigo TAKASAWA
Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Asahimachi, Machida-shi, Tokyo 194, Japan
*Research and Development Division, Kyowa Hakko Kogyo Co., Ltd., Otemachi, Chiyoda-ku, Tokyo 100, Japan
Received February 19, 1985

The development of agar plate screening techniques has allowed the isolation of mutants of Trichoderma reesei capable of synthesizing cellulase under the conditions of a high concentration of glucose. Mutants resistant to catabolite repression by glycerol or glucose were isolated on Walseth's cellulose (WC) agar plates containing 5% glycerol or 5% glucose, respectively. Mutants resistant to catabolite repression by glycerol were not derepressed enough for the production of cellulase on WC agar plates containing 5% glucose or in flask cultures with a mixture of 1% Avicel and 3% glucose. On the contrary, two mutant strains resistant to catabolite repression by glucose (KDD-10 and DGD-16) produced large clearing zones on WC agar plates containing 5% glucose. Both strains could begin to produce CMCase even in the presence of residual glucose and finally produced 1.5 times the CMCase activity, in flask cultures on 1% Avicel and 3% glucose, than that with 1% Avicel alone. These results suggest that KDD-10 and DGD-16 are comparatively derepressed by glucose for cellulase production.

The industrial use of cellulase for the conversion of cellulose to glucose has been handicapped by the relatively high cost of cellulase. Therefore, it seemed useful to obtain microorganisms with high cellulase producing abilities. Among cellulolytic microorganisms, T. reesei is known to be the highest producer of cellulase, and many reports have appeared on T. reesei mutants capable of hyperproduction of cellulase. We also began to improve the cellulase producing ability of T. reesei KY 746, which was derived from QW 9414 by monoclonal isolation, and developed a semiquantitative plate assay method for selecting fungal mutants showing hyperproduction of cellulases.

In order to lower the cost of cellulase, inexpensive carbon sources are also necessary for cellulase production. We think that molasses, extensively used in the fermentation industry, and agricultural wastes, such as whey, rice straw and bagasse, may be available for cellulase production. Glucose and other easily metabolizable sugars inhibit the formation of cellulase by the mechanism of catabolite repression. When a large amount of molasses, which contains high concentrations of glucose and fructose, is used for cellulase production, catabolite repression will occur. To overcome these problems, cellulase mutants resistant to catabolite repression have been sought, and among those found MCG-77 (Natick) and C-30 (Rutgers) have been well investigated. When these mutants were used for cellulase production in liquid cultures, the addition of only 1% glucose to the cellulose medium or to the 1% cellobiose medium instead of 1% cellulose was made. But, there has been no report on the cellulase production in a liquid culture under the conditions of a high concentration of glucose such as in the case of the addition of 3% glucose to a cel-

† Production of Ethanol from Biomasses. Part II.
lulose medium. We are also trying to obtain T. reesei mutants which are resistant to catabolite repression and to use them for practical application. The present paper describes a screening method for mutants resistant to catabolite repression by glycerol or glucose and their cellulase productivities under the fermentation conditions of a high concentration of glycerol or glucose.

MATERIALS AND METHODS

**Microorganism.** T. reesei strain KY 746, which was selected by monoclonal isolation from Natick mutant strain QM 9414, was used as a starting strain for mutation. The series of mutational steps is shown in Fig. 1. MCG-77 (Natick mutant) was used as the control in the catabolite repression experiments.

**Mutagenesis and enrichment procedure.** Fresh spores suspended in saline (10^7 to 10^8/ml) were used for mutagenesis. They were subjected to UV irradiation to give an about 1% survival rate or N-methyl-N'-nitro-N-nitrosoguanidine (NTG) treatment to give an about 50% survival rate, and then incubated at 30°C for 7 days on potato dextrose agar plates. The colonies that grew on these plates were transferred to WC agar plates containing 1~5% glycerol or 1~5% glucose followed by incubation at 30°C for 3 days. For these plates containing 1% WC, 0.4% l-sorbose and 0.1% Triton X-100 were used to restrict the spread of hyphae of T. reesei as previously reported. Other components of these plates were also given previously. Cellulase yields on plates containing these media were determined as the clearing zone ratio (clearing zone diameter/colony diameter).

**Submerged fermentation conditions.** Submerged fermentation studies were carried out at 28°C using the modified Mandels's medium described previously except for the concentration of (NH₄)₂SO₄ (5.6 g/liter) and that the pH was controlled at pH 4.0 by addition of 100 mM tartrate buffer. As a substrate causing catabolite repression, 1~3% glycerol or 1~3% glucose was added to the cellulose medium. In each 300 ml Erlenmeyer flask (working volume; 50 ml), spores from V-8 agar slants were incubated. The supernatant of the cultural broth was analyzed for enzymatic activity and the residual amount of glycerol or glucose.

**Enzyme and sugar determinations.** Cellulase activities in the culture fluid were examined by the modified method of Mandels et al. Sodium carboxymethyl cellulose was used as a substrate (CMCase). The reducing sugar released was determined by the Somogyi-Nelson method. One unit of enzyme activity was expressed as the international unit. The residual amount of glucose or glycerol was determined with a Nihon Bunko Trirotor III HPLC apparatus equipped with a refractive index detector and a Shodex Ionpack C-811 column, with 0.1% H₃PO₄ as an eluant.

RESULTS AND DISCUSSION

**Mutational steps**

The series of mutants from T. reesei KY 746 is presented in Fig. 1. The screening procedure and properties of the mutants, such as N-25, N-34, K-14, J-37, KDR-11 and KDR-27, were previously reported. These mutants were higher cellulase producers than KY 746. Furthermore, the KDG-series and JG-series (mutants resistant to catabolite repression by glycerol) were isolated from KDR-27 and J-37 by NTG treatment, respectively. The KDD-series and DGD-series (mutants resistant to catabolite repression by glucose) were isolated from KDR-11 and KDG-12, which is a representative strain of the KDG-series, by NTG treatment, respectively.

**Cellulase production of mutants resistant to catabolite repression by glycerol**

Although there have been many reports of T. reesei mutants, such as NG-14 and MCG-77, resistant to catabolite repression by glycerol, a detailed study for the application of glycerol to cellulase production has not appeared. To demonstrate the glycerol effect in
Mutants Resistant to Catabolite Repression of *T. reesei*

<table>
<thead>
<tr>
<th>Table I. CELLULASE PRODUCTION by <em>T. reesei</em> Mutants in Plate Cultures in the Presence of Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>KY 746</td>
</tr>
<tr>
<td>J-37 (Parent)</td>
</tr>
<tr>
<td>JG-101 (Mutant)</td>
</tr>
<tr>
<td>KDR-27 (Parent)</td>
</tr>
<tr>
<td>KDG-12 (Mutant)</td>
</tr>
<tr>
<td>MCG-77</td>
</tr>
</tbody>
</table>

a — , a clearing zone did not appear.
Abbreviations: WC, Walseth’s cellulose; Gly, glycerol.

<table>
<thead>
<tr>
<th>Table II. CELLULASE PRODUCTION by <em>T. reesei</em> Mutants in Flask Cultures in the Presence or Absence of Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>KY 746</td>
</tr>
<tr>
<td>JG-101</td>
</tr>
<tr>
<td>KDG-12</td>
</tr>
<tr>
<td>MCG-77</td>
</tr>
</tbody>
</table>

The flask cultures were carried out for 8 days and other conditions were given in MATERIALS AND METHODS.
Abbreviations: Avi, Avicel; Gly, glycerol.

Cellulase production, WC agar plate cultures with 1 ~5% glycerol were carried out at 30°C for 3 days (Table I). Although many mutants formed large clearing zones on the plates containing 1% glycerol, the addition of 5% glycerol to the plates restricted the formation of clearing zones by these mutants. KDG-12 derived from KDR-27, and JG-101 from J-37 were selected for the resistance to catabolite repression by glycerol. Because their clearing zone ratios were larger than that of MCG-77 on plates containing 5% glycerol, they were relatively resistant to catabolite repression by glycerol. In a previous report, we showed the correlation between the clearing zone on a WC agar plate without glycerol or glucose and the CMCase production in a flask culture with Avicel for *T. reesei* mutants. We expected that there was the same correlation even in the presence of glycerol.

In order to investigate the CMCase production of KY 746, JG-101, KDG-12 and MCG-77, flask cultures with 1% Avicel plus 0 ~3% glycerol media were carried out (Table II). The addition of glycerol to the medium brought about a decrease in the productivity of cellulase in all mutants except JG-101. Furthermore, in the cultures with 1% Avicel plus 3% glycerol, the amounts of residual glycerol on the 8th day of cultivation for KY 746, JG-101, KDG-12 and MCG-77 were 3.0 mg/ml, 9.9 mg/ml, 9.0 mg/ml and 20.0 mg/ml, respectively. Since the cellulase productivity decreased on addition of glycerol and the efficiency of utilization of glycerol was low, the application of glycerol to cellulase production by *T. reesei* mutants seemed to be difficult.

**Cellulase production of mutants resistant to catabolite repression by glucose**

There has been no attempt to obtain mutants resistant to catabolite repression by glucose by direct plate screening on glucose medium. In many studies, such mutants were obtained indirectly by selection for resistance to 2-deoxyglucose or to catabolite repression by glycerol. To elucidate the repression effect of glucose in the mutants resistant to catabolite repression by glycerol, WC agar
plate cultures in the presence of 1~5% glucose were performed (Table III). In the plate cultures with 5% glucose, KDG-12 alone formed a clearing zone, which was only a small one, among our mutants. Therefore, KDG-12 was partially resistant to catabolite repression by glucose.

Isolation of mutants resistant to catabolite repression by glucose was carried out directly by plate cultures with 5% glucose. The rate of appearance of such mutants was very low compared with that of mutants resistant to catabolite repression by glycerol. KDD-10 and DGD-16 were each selected from about 100,000 colonies after NTG treatment of KDR-11 and KDG-12, respectively. They formed large clearing zones on plates containing 5% glucose (Table III).

The mutants resistant to catabolite repression by glycerol or glucose were tested in flask cultures with the 1% Avicel plus 3% glucose medium compared with cultures with the 1% Avicel medium. The time course of CMCase production and assimilation rate of glucose are shown in Fig. 2. All the cultures reached the maximum level on the 8th day of cultivation. KY 746 did not produce CMCase in the 1% Avicel plus 3% glucose medium until glucose had been completely consumed and the subsequent resting period (2 days) had elapsed. The CMCase activity of KY 746 reached the same level in both media, 1% Avicel and 1% Avicel plus 3% glucose, on the 8th day of

Fig. 2. Cellulase Production by T. reesei Mutants in Flask Cultures with the 1% Avicel Plus 3% Glucose Medium and the 1% Avicel Medium, and the Assimilation Curve for Glucose in the 1% Avicel Plus 3% Glucose Medium.

- , CMCase in the 1% Avicel plus 3% glucose medium; O-O, CMCase in the 1% Avicel medium; ---, residual amount of glucose in the 1% Avicel plus 3% glucose medium.

a) KY 746; b) KDG-12; c) DGD-16; d) KDD-10; e) MCG-77.

Fig. 3. Cellulase Production by T. reesei Mutants in 5 Liter Jar Fermentors with the 6% Avicel Medium.

The detailed culture conditions were given previously.15) - , KY 746; O-O, KDD-10; ▲▲, MCG-77.
culture. The rate of consumption of glucose by KDG-12 was the highest among the examined mutants. Its CMCase production began at a comparatively early time, but it reached almost the same level in the 1% Avicel plus 3% glucose medium as in the 1% Avicel medium. The time course for KDG-12 resembles that for MCG-77. On the contrary, KDD-10 and DGD-16 began to produce CMCase even in the presence of glucose at the beginning of the cultivation. On the 8th day of cultivation, the CMCase activities of KDD-10 and DGD-16 in the 1% Avicel plus 3% glucose medium had reached approximately 1.5 times those in the 1% Avicel medium. These results showed that the cellulase production of KDD-10 and DGD-16 was greater than that of MCG-77 under the conditions of a high concentration of glucose.

In many studies,\textsuperscript{2,4,12} mutants resistant to catabolite repression showed enhanced cellulase productivity in cellulose medium. Three mutants of \textit{T. reesei}, KY 746, KDD-10 and MCG-77, were grown in 5 liter jar fermentors with 6% Avicel (Fig. 3). Because the cultural conditions in 5 liter jar fermentors could be regulated for the best growth of \textit{T. reesei} mutants (pH, 4.0, aeration, 1 VVM, agitation, 450 rpm), effective cellulase production with a high cellulose concentration medium will be possible. KDD-10 showed the fastest rate of enzyme production among these mutants. Thus, KDD-10 has resistance to catabolite repression by glucose and very high cellulase productivity.

\textit{Acknowledgments.} This work was supported by the funds of the Research Association for Petroleum Alternative Development. We also gratefully acknowledge the skillful technical assistance of Miss Hiroko Nodake.

\textbf{REFERENCES}