CELLULASE PRODUCTION BY A PARTIALLY CATABOLITE-RESISTANT MUTANT OF TRICHODERMA REESEI

S. K. RAKSHIT* AND V. SAHAI

Biochemical Engineering Research Centre, Indian Institute of Technology,
New Delhi–110 016, India

(Received September 18, 1989)

The new mutant *Trichoderma reesei* E-12 was better than the parent strain QM 9414 and strain D1/6 in terms of cellulase productivity. Due to its greater resistance to catabolite repression there was scope for further improvement of enzyme productivity by better control of the environment inside the bioreactor. Mutant E-12 requires higher nitrogen concentration than other strains. A peptone concentration of 0.2% minimizes the foaming problem and the drop in filter paper activity at the decreased peptone level is not significant. The mutant strain has a potential for enhanced cellulase biosynthesis with better environmental control strategies.

A variety of strategies have been tried to convert the abundantly available lignocellulosic residues into useful chemical feedstock. The high moisture content of these residues makes them less suitable for most chemical treatments and more suitable for enzymatic hydrolysis. The major bottleneck to industrial scale application of the latter method is the high cost of cellulase enzyme. Physical mutation programs are under way at a number of laboratories to isolate a mutant capable of hyper-producing the enzyme complex. The aim of the work reported here was to compare a few mutants screened at our laboratory. The possibility of the best strain being resistant to some intracellular constraints to oversynthesis of cellulase was then investigated. The medium was modified for the new mutant so that conditions could be standardized to optimize control variables.

The development of a practical process for enzyme production is based mainly on the *Trichoderma reesei* fungal system. The biosynthesis of cellulase occurs under various strict genetic and biochemical control mechanisms. Intracellular economy constrains the organism’s oversynthesis of enzymes and other metabolites unless their production is useful to growth and survival. The controls affecting cellulase

* Present address of author to whom reprint requests to: Department of Chemical Engineering, Indian Institute of Technology, Madras-600 036, India.
biosynthesis include induction by insoluble cellulose, and catabolite and endproduct repression by glucose and cellobiose. So a good microbial mutation program attempts to isolate organisms that are no longer subject to these constraints. A number of Trichoderma reesei strains were screened after physical mutation procedures (Fig. 1) at the Biochemical Engineering Research Centre, I.I.T., Delhi. The objective was to compare the activity of the new mutant E-12 with the parent strain QM 9414 and with another new mutant D1/6 isolated earlier at the Centre. Experiments were then carried out to see if the new mutant produced the enzyme better because of the breakdown of any control mechanism. Medium composition was then sought suitably modified for the new mutant so that the environmental parameters (pH, temp., etc.) could be identified and the critical control variables could be rigorously optimized.

MATERIALS AND METHODS

Organisms. Trichoderma reesei QM 6a was the parent strain initially used by many workers for the biosynthesis of the cellulase enzyme complex. A chronology of mutant strains developed in different laboratories is given in Fig. 1. Mutants QM 9414, D1/6 and E-12 were used in our work.

Fermentor. All experiments were carried out in a 4-l working volume
Bioengineering AG fermentor. As a precaution against spores being present in the cellulose, the fermentor was steam sterilized (121°C, 30 min) on two consecutive days, first with only cellulose slurry and the second time with all components of the medium. Cellulose levels of 6 and 1%, with corresponding changes in the nitrogen content, were used in different experiments. Temperatures were maintained at 30°C for the first 24 h and 28°C for the rest of the fermentation. pH was controlled by adding 2 N HCl/2 N NaOH.

**Media composition.** Microcrystalline cellulose powder (MCCP) purified from native cotton was obtained from the V.P. Chest Institute, Delhi, India. The basal medium suggested by Mandels and Reese (1) was modified for the fermentor studies to facilitate better pH control. The initial carbon to nitrogen ratio of eight was maintained in all runs, unless otherwise noted.

**Development of inoculum.** To acclimatize the cultures to high levels of cellulose the inoculum was developed in two stages. In the first stage a medium containing 0.5% cellulose and 1% glucose was inoculated using a fresh slant of T. reesei. The culture obtained was used to inoculate the seed stage shake flask medium containing 1% cellulose and 0.5% glucose. Sufficient seed culture was added to the fermentor to make the biomass level about 1.8 to 2 mg/ml in the fermentor.

**Analytical procedures.** Estimation of the reducing sugars in the broth was estimated using the dinitrosalicylic acid (DNS) method (2) and the Nelson Somogyi method (3, 4).

The cell mass in the presence of cellulose was estimated by first filtering the broth. The residual cellulose-biomass mixture was repeatedly boiled with 2 N sodium hydroxide and the protein content was then measured by the Micro-Biuret method (5).

The method recommended by I.U.P.A.C. for measuring cellulase activities (6) was used in the enzyme assays.

**RESULTS AND DISCUSSION**

**Comparative study of mutants**

The *Trichoderma reesei* mutants E-12 were compared initially with D1/6 and QM 9414 on 6% cellulose medium. It has been reported (7) that with cellulose levels above 1%, the pH of the medium drops below 2.0 resulting in inactivation and autolysis of the organism. To prevent this, the pH was not allowed to drop below 3.5 in runs with any of three mutants. The mutants took different time periods before the pH began to fall and the biomass reached a maximum (Figs. 2-4). Measurable quantities of cellulase, in terms of filter paper activity was available only after the growth rate was zero or negative. The results given in Table 1 indicate that D1/6 and E-12 produced 25% more filter paper activity than QM 9414. The maximum achievable activity was comparable for D1/6 and E-12, but the latter synthesized the enzyme faster. So E-12 had a higher productivity than any other available strain.
Resistance to catabolite repression caused by reducing sugars

The data in Table 1 indicates the superiority of strain E-12 over D1/6. But this was not considered adequate for the selection of the best strain, because a given strategy of pH control that yields more enzyme by mutant E-12 may not be as good for the biosynthesis by D1/6. There could be a better pH profile which would enhance the rate of enzyme production. Starting with the same amount of inducer, 6% cellulose, this will depend on the ability of the strain to resist catabolite repression of enzyme synthesis resulting from the increased level of reducing sugar that is likely to occur when better pH profiles are implemented in a fermentor using these strains.
Experiments were therefore performed to observe the effect of reducing sugar level on the cellulase biosynthesis. To cultures of D1/6 and E-12, separately grown on 1% cellulose, a pulse of glucose was given at 70 h when measurable quantities of cellulase were available in the extracellular broth. The reducing sugar level was increased to 500 mg/ml (Figs. 5 and 6).

Strain E-12 appeared to resist catabolite repression to a greater extent than D1/6. It resumed enzyme synthesis at a reducing sugar level of 300 mg/ml or less while D1/6 resumed the synthesis only when the reducing sugar had gone below 120 mg/ml. More than 70 mg/ml of reducing sugar has been reported to cause repression in QM 9414 (Sahai, V., Ph. D. Thesis, IIT, Delhi, India, 1978).

With the strategy of control which did not permit the pH to fall below 3.5, the maximum reducing sugar available in the fermentor was 120 mg/ml starting with 6% cellulose in the medium for mutant D1/6. In this mutant there was little scope for increasing productivity by implementing a pH profile which would have
enhanced the hydrolysis of cellulose, since the reducing sugar level during the fermentation was already nearly critical and would have resulted in repression even though more biomass could be formed. However in E-12 there was sufficient scope for further enhancement of enzyme productivity as the reducing sugar level was 150 mg/ml in a batch culture. Considering the increased resistance to catabolite...
repression, the wider scope for further enhancement of enzyme productivity by better manipulation of pH, and other parameters, a preference for strain E-12 appeared to be justified.

**Effect of organic nitrogen on cellulase production**

Before environmental conditions could be optimized, we needed to determine the nitrogen requirement of the new mutant. Both the type and level of nitrogen had to be optimized.

Mandels (8) had reported that organic nitrogen (peptone) should be included at one-tenth the level of cellulose. With 6% cellulose, 0.6% peptone would be required. But with such levels of peptone, we encountered severe foaming problems in the fermentor. The problem was aggravated because of the required aeration and agitation conditions. The high cost of peptone also had to be considered. It was thus decided to determine the minimum level of peptone to be included in the medium which would minimize the foaming problem but would not significantly decrease filter paper activity at the lower peptone level.

To study the effect of the organic nitrogen source on enzyme production, the amount of peptone and inorganic nitrogen as ammonium sulphate, was varied to make the initial carbon to nitrogen ratio eight in the media. Various levels of peptone were used in the different experiments making up the remaining nitrogen requirement with the ammonium sulphate. In the presence of peptone there was an initial increase in pH due to consumption of amino acids. After all the peptone was consumed, the pH began to fall. When the medium contained no peptone, the pH dropped soon after inoculation, because in the absence of peptone the organism used ammonium sulphate as the nitrogen source. This caused the hydrogen ion in the medium to accumulate and the pH dropped to 3.5 within 2 to 3 h. Due to continuous low pH from the beginning, both growth and enzyme synthesis were very poor. However, in another medium containing no peptone, the pH was prevented from falling below 5.2 for the first 24 h after inoculation. The biomass level in this run was almost as high as the runs with peptone (Table 2) but enzyme yields were reduced. Peptone acts as a source of a certain amino acid which appears

<table>
<thead>
<tr>
<th>Run Nos.</th>
<th>Peptone level (%)</th>
<th>Foaming problem</th>
<th>Maximum F. P. activity (I.U./ml)</th>
<th>Overall productivity (I.U./h; 168h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10*</td>
<td>0</td>
<td>Slight</td>
<td>4.0</td>
<td>23.80</td>
</tr>
<tr>
<td>11</td>
<td>0.1</td>
<td>Controllable</td>
<td>5.0</td>
<td>29.76</td>
</tr>
<tr>
<td>12</td>
<td>0.2</td>
<td>Controllable</td>
<td>6.8</td>
<td>40.47</td>
</tr>
<tr>
<td>13</td>
<td>0.4</td>
<td>Uncontrollable</td>
<td>7.2</td>
<td>42.85</td>
</tr>
<tr>
<td>14</td>
<td>0.6</td>
<td>Uncontrollable</td>
<td>7.7</td>
<td>45.83</td>
</tr>
</tbody>
</table>

* pH controlled not to fall below 5.2 for 24h.
to enhance enzyme yields. The reduction in the yield of enzyme in cultures with no peptone can probably be attributed to the lack of such amino acids. A complete replacement of peptone, though desirable for foam control, results in a 50% reduced yield of enzyme. However at 0.2% peptone, foaming was controllable and the yield was only 10% less than with the 0.6% peptone run. Peptone at 0.2% level appears to be most suitable from the culture operation point.

*Effect of initial carbon to nitrogen (C/N) ratio in batch cellulase production*

With a high level of cellulose in the medium nitrogen may be limited if sufficient peptone and ammonium sulphate are not added to the system. At 6% cellulose, nitrogen limitation has been reported if the C/N ratio is greater than eight for QM 9414 (7,8) and for D1/6 (Manihar, R., M. Tech. Thesis, IIT, New Delhi, India, 1982). The amount of nitrogen required by a particular mutant depended on the amount of cellular and extra cellular protein synthesized. The initial C/N ratio had to be such that growth and enzyme synthesis were not hindered by a deficiency of either the carbon (cellulose) or the nitrogen (ammonium sulphate and peptone).

![Graph](image)

**Fig. 7.** Prediction of nitrogen limitation from NaOH addition data in T. reesei E-12 fermentation. Dotted line indicates the amount of NaOH needed for neutralization of theoretical maximum milliequivalents of H₂SO₄ that could be released. Experiments with C/N in the range 6.5–8.5.
As a nitrogen limited condition would have lead to considerable loss of enzyme activity, it was decided to study the effect of this parameter on the new mutant E-12. Experiments were run with a C/N ratio of 6.5, 7.0, 7.5, 8 and 8.5.

The amount of inorganic nitrogen source, ammonium sulphate, taken up by the organism was estimated by the amount of sodium hydroxide taken up by the system to control the pH at 3.5 after a natural fall. This was compared to the theoretically calculated amount of sodium hydroxide that would be taken up if all of the ammonium sulphate available were consumed (Fig. 7). For cultures where the amount of sodium hydroxide required by the culture equalled this calculated maximum value, there was a good possibility of nitrogen limitation in the medium. An initial carbon to nitrogen ratio of eight and above was found to lead to such a limitation. But a C/N ratio of 7.5 was sufficient.

The enzyme activity profiles with the two initial levels of C/N ratio (7.5 to 8.0) indicate (Fig. 8) a drop in the enzyme production rate late in the fermentation in runs with a C/N ratio of 8.0, due to the lack of nitrogen. As the same amount of cellulose was used in all experiments, the results indicated that mutant E-12 required more nitrogen for the soluble protein synthesis that was released into the extra-cellular media late in the fermentation.

REFERENCES

2) Summer, J. R. and Somers, G. F., Laboratory Experiments in Biological Chemistry, Academic

3) Nelson, N., A photometric adaptation of the Somogyi method for the determination of glucose. 


6) Ghose, T. K., Final recommendation on the measurement of cellulase activities, Biotechnology 

7) Mandels, M. and Andreotti, R. E., Problems and challenges in the cellulose to cellulase 

8) Mandels, M., Measurement of cellulase activity, in the Second International Course in 
   Bioconversion and Biochemical Engineering, New Delhi, India (1980).