0273-2289/92/3435—0175$02.00

Improvement of Cellulase Activity in *Trichoderma*

D. K. SANDHU* AND SONYA BAWA

School of Life Sciences, Guru Nanak Dev University, Amritsar, India

ABSTRACT

The conditions for the production and regeneration of protoplasts from *Trichoderma reesei* QM 9414 were optimized. Mutants obtained from *Trichoderma reesei* QM 9414 were crossed, using protoplast fusion. In the crosses attempted, the diploids showed enhanced yields of exoglucanase, whereas the yield of endoglucanase and β-glucosidase was intermediate as compared to their respective parents. In some of the segregants, the yields were further enhanced, indicating the use of protoplast fusion techniques in developing breeding strategies for strain improvement. The study of cellulase mutants as such and the segregants revealed that the three cellulase components appear to be regulated independently of each other.

Index Entries: *Trichoderma;* cellulase; protoplast fusion; strain improvement.

INTRODUCTION

The *Trichoderma* species are well known as cellulase producers (*1*). However, *Trichoderma* lacks sexuality (*2,3*), and to gain knowledge of its genetics, recombinant strains can be produced by induced heterokaryosis via protoplast fusion. The latter is a very useful tool for genetic analysis and can be exploited for registering significant advances in strain improvement, as well as the production of novel strains with novel properties, produced as a result of interaction of known biochemical pathways in altered environments (*4,5*). In the present study, the potential of protoplast fusion for genetic studies and strain improvement was utilized with a view toward selecting recombinants with altered or improved cellulase character.

*Author to whom all correspondence and reprint requests should be addressed.

Table 1
Characteristics of *Trichoderma reesei* QM 9414 and Its Mutants

Mutant	Requirement	Cellulase activity, IU mL^{-1}[a]		
		Exoglucanase	Endoglucanase	β-glucosidase
QM 9414	Prototroph	1.42	9.05	0.27
Tr84	*thr, leu*	0	0.05	0.08
Tr48	*thr, arg*	0.25	4.36	0.22
Tr162	*ade, lys, gal*	0.03	6.03	0.19

[a]In the three auxotrophs, exoglucanase activity was assayed on the eighth day, whereas endoglucanase and β-glucosidase yields were noted on 12th day, which were days of maximum enzyme production in QM 9414.

MATERIALS AND METHODS

Organisms

Trichoderma reesei QM 9414 (Natick Laboratories, USA), a hypercellulase producer, and mutants derived from it were used in the present study. The mutants were indistinguishable morphologically, i.e., all produced green conidia. Their auxotrophy and cellulase production are given in Table 1. The cultures were maintained in soil at 4°C, and whenever required, subculturing was done on complete medium (CM) (glucose, 10 g; KH_2PO_4; 10 g; yeast extract, 3 g; agar 20 g/L) slants.

Protoplast Production, Fusion, and Segregant Analysis

For protoplast production from spores, optimized conditions of enzyme and conidial concentration, time and medium for preincubation, type and concentration of osmotic stabilizer, temperature, pH, and age of culture obtained, by modifying the method of Bos and Slakhorst (*6*) were used: Various combinations of different stabilizers and their concentrations in different media were assayed in order to get maximum regeneration (Table 2). Protoplast fusion was performed according to the procedures described by Bos et al. (*7*). Equal numbers of protoplasts from the two auxotrophic strains were mixed together, centrifuged, and resuspended in 1 mL of freshly prepared polyethylene glycol solution (35% w/v PEG, 4000 mol. wt; 50 m*M* $CaCl_2$). One milliliter of 0.6*M* KCl was added after incubating the mixture at 30°C for 20 min. Aliquots (0.1 mL) were plated on minimal medium (complete medium without yeast extract) supplemented with common amino acid requirement of the two parents and stabilized with 0.4*M* $(NH_4)_2SO_4$ to isolate the potential diploids. Haploidization was induced with *para*-fluorophenylalanine (PFPA) and benlate. The segregating sectors were isolated and subjected to analysis.

Table 2
Optimal Conditions for Regeneration of Protoplasts
Isolated from Spores of *Trichoderma reesei* QM 9414

Medium	Stabilizer present in the medium		Stabilizer present in the suspending solution: KCl 0.4*M*	KCl 0.6*M*	$(NH_4)_2SO_4$ 0.4*M*	$(NH_4)_2SO_4$ 0.6*M*
CMI[a]		None	10.14	0.00	0.00	4.30
	KCl	0.4*M*	0.57	45.14	0.14	0.00
	$(NH_4)_2SO_4$	0.6*M*	19.14	23.40	1.70	0.00
	$(NH_2)_2SO_4$	0.4*M*	22.00	13.40	10.50	6.59
		0.6*M*	24.85	53.42	2.28	0.00
CMII[b]		None	0.00	18.57	0.00	0.00
	KCl	0.4*M*	56.75	61.71	6.00	14.00
		0.6*M*	49.14	31.40	0.00	10.57
	$(NH_4)_2SO_4$	0.4*M*	68.28	89.70	19.40	10.57
		0.6M	71.42	71.70	14.00	6.57

[a]CMI: Glucose, 10 g; yeast extract, 3 g; KH_2PO_4, 10 g/L.
[b]CMII: Glucose, 10 g; yeast extract, 30 g/L.

Estimation of Cellulase Activity

The cellulase was measured in terms of exoglucanase, endoglucanase, and β-glucosidase (*8*), by growing the organism in CM broth with 1% microcrystalline cellulose as the carbon source. Enzyme activity has been expressed in international units (IU), as the amount of enzyme needed to release 1 μmol of glucose or *p*-nitrophenol. In case of segregants, where a large number of isolates had to be processed, the cellulose agar clearing test for exo- and endoglucanase and color production for β-glucosidase were employed (*9*). The clearing zone was measured as the diameter of the clearing zone, including the colony, and then subtracting the colony diameter.

Genetic Crosses

Two crosses were made, one between Tr48 and Tr84, and the other between Tr84 and Tr162.

RESULTS

Protoplast release was maximum (9.5×10^5 mL^{-1}) when 1×10^6 spores mL^{-1} from 38-h-old cultures were preincubated for 1 h and then treated with 8 mg/mL^{-1} Novozym for 4.5 h at 28°C in medium containing 0.4*M* $(NH_4)_2SO_4$ as osmotic stabilizer.

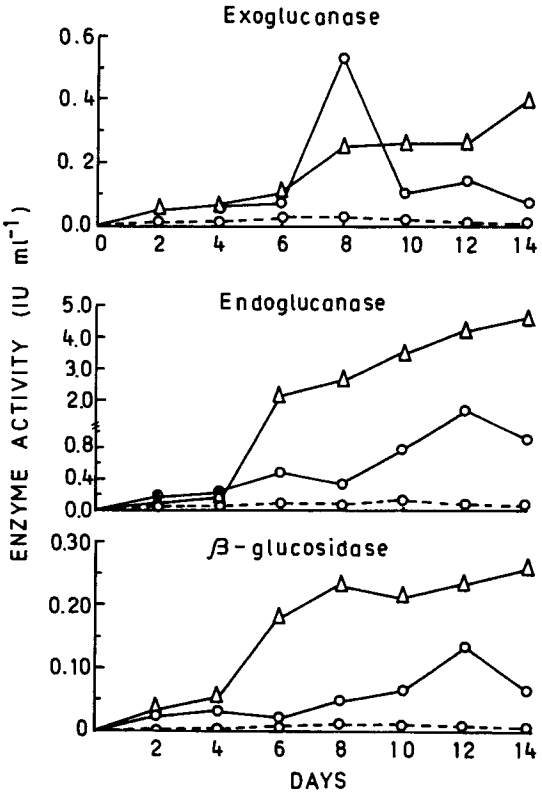

Fig. 1. Cross A. Cellulase enzyme activities of Tr84, ○--○, Tr48, △—△, and their diploid A, ○—○.

$$\text{Cross A: } \frac{\text{Tr84 thr}^- \text{ leu}^- \text{ arg}^+ \text{ exo}^- \text{ endo}^- \beta\text{-glu}^-}{\text{Tr48 thr}^- \text{ leu}^+ \text{ arg}^- \text{ exo}^+ \text{ endo}^+ \beta\text{-glu}^+} \quad (1)$$

Quantitative assessment of cellulase production revealed enhanced exoglucanase (0.52 IU mL^{-1}), but intermediate endoglucanase and β-glucosidase activity by the diploid as compared to its parents (Fig. 1).

Following PFPA-induced segregation, 49 segregants were recovered, which when assayed qualitatively for exoglucanase, endoglucanase, and β-glucosidase gave 47, 32, and 46 producers, respectively. Quantitative studies on 20 randomly selected segregants gave ratios of enyzme producers to nonproducers of: exoglucanase 3:1, endoglucanase 1:0, and β-glucosidase 4:1. The producer segregants showed a range of enzyme titers (Fig. 2). The mean titer of producer progeny was more than that of the producer parent in case of exoglucanase and β-glucosidase, whereas it was less than the producer parent for endoglucanase.

$$\text{Cross B: } \frac{\text{Tr84 thr}^- \text{ leu}^- \text{ ade}^+ \text{ lys}^+ \text{ gal}^+ \text{ exo}^- \text{ endo}^- \beta\text{-glu}^-}{\text{Tr162 thr}^+ \text{ leu}^+ \text{ ade}^- \text{ lys}^- \text{ gal}^- \text{ exo}^+ \text{ endo}^+ \beta\text{-glu}^+} \quad (2)$$

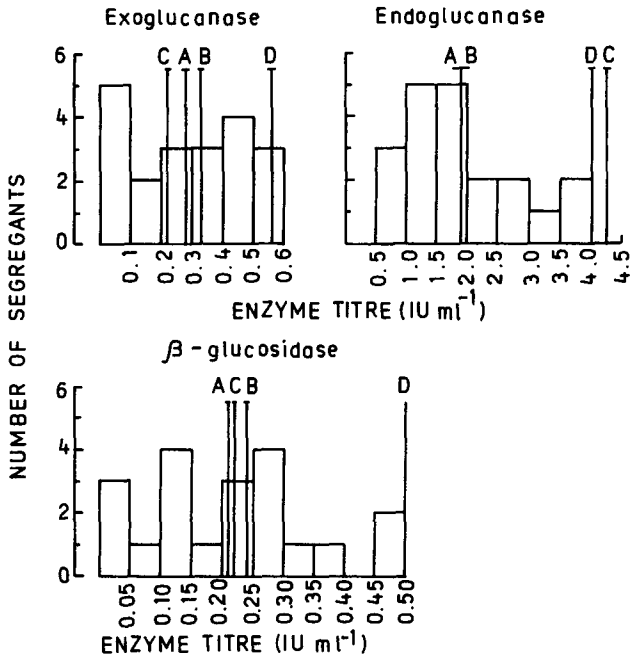

Fig. 2. Cross A. Cellulase production by recombinants from diploid A. Vertical lines mark: A, mean of all progeny, B, mean of cellulase producing progeny, C, titer of producing parent Tr48, and D, maximum titer of a segregant.

The pattern of comparative cellulase production by parents and the diploid in Cross B was similar to that in Cross A. Intermediate values of endoglucanase and β-glucosidase were noted. Exoglucanase activity of diploid showed an early peak, which was equated only at later stages by the producer parent, Tr 162 (Fig. 3).

Qualitative assay of the 81 segregants isolated revealed 41, 29, and 81 producer segregants for exoglucanase, endoglucanase, and β-glucosidase, respectively. Quantitative data of enzyme activity from 18 randomly selected segregants gave the ratio of producers to nonproducers as exoglucanase 1:1, endoglucanase 1:0, and β-glucosidase 1:1. In Cross B, mean enzyme titer of producer progeny was less than the titer of producer parent for all the three components (Fig. 4).

Among the 38 segregants selected from both crosses, 13 registered significant increase in the yield of either of the cellulase components. Of these segregants, AS 11 (0.56 IU mL^{-1}), BS5 (7.4 IU mL^{-1}), and AS 29 (0.50 IU mL^{-1}) showed the maximum exoglucanase, endoglucanse, and β-glucosidase activities, respectively (Table 3).

The enzyme activities in extracts from the parental and diploid strains are shown in Table 4. In the case of exoglucanase, enzyme activity in the diploid extract was more than the arithmetic mean predicted for equal expression of structural genes, whereas in endoglucanase and β-glucosidase, the enzyme activity was less than that observed in haploid mutant stains.

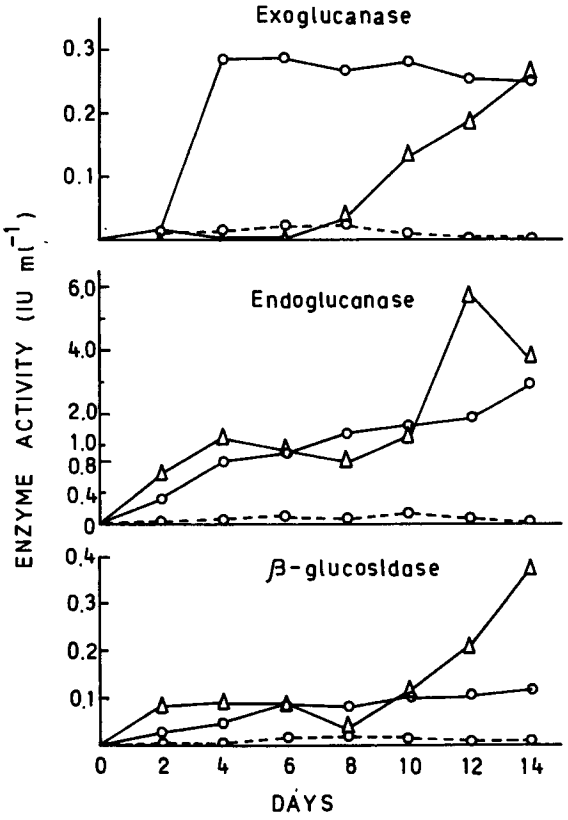

Fig. 3. Cross B. Cellulase enzyme activities of Tr84, O--O, Tr162, △—△, and their diploid B, O—O.

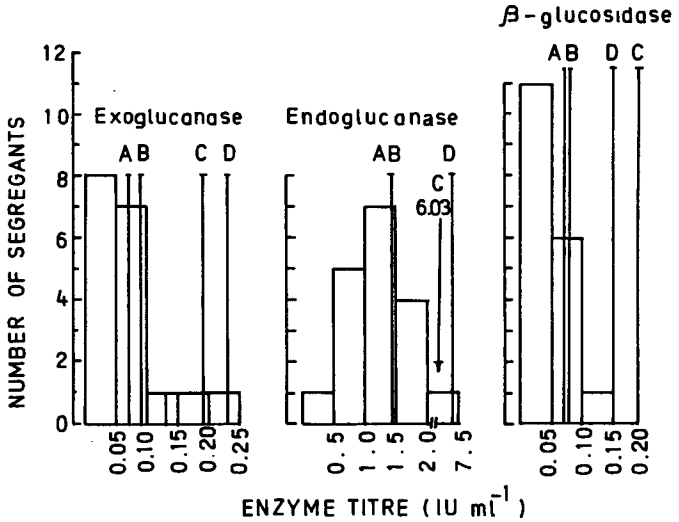

Fig. 4. Cross B. Cellulase production by recombinants from diploid B. Vertical lines mark: A, mean of all progeny, B, mean of cellulase producing progeny, C, titer of producing parent Tr162, and D, maximum titer of a segregant.

Table 3
Yield of Cellulase by the Parental Strains
(Tr48 and Tr162) and Some Recombinants

Strains	Enzyme production, IU mL^{-1}
Mutants	Exoglucanase
Tr48	0.22
Tr162	0.19
Segregants	
AS 11	0.56
AS 8	0.53
AS 10	0.50
AS 4	0.48
AS 18	0.48
AS 16	0.43
AS 40	0.41
AS 7	0.40
Mutants	Endoglucanase
Tr48	4.36
Tr162	6.03
Segregant	
BS 5	7.4
Mutant	β-glucosdiase
Tr48	0.22
Tr162	0.19
Segregant	
AS 29	0.50
AS 4	0.46
AS 36	0.40
AS 16	0.33

Table 4
Enzyme Production by the Haploids and Their Diploids

Mutant strain	Haploid Enzyme production	Diploid[a] IU/mL	Percent arithmetic mean
	Exoglucanase		
Tr48	0.25	0.53	>100
Tr162	0.03	0.27	>100
	Endoglucanase		
Tr48	4.36	1.64	74
Tr162	6.03	1.90	62.5
	β-Glucosidase		
Tr48	0.22	0.13	87
Tr162	0.19	0.10	74

[a]Diploids were constructed by crossing each mutant with mutant strain 84, the activity of which is exoglucanase = 0, endoglucanase = 0.05, and β-glucosidase = 0.08 IU/mL.

DISCUSSION

In Tr84, all three cellulase components were highly repressed indicating a regulatory gene mutation, whereas in Tr48 and Tr162, only the exoglucanase gene appeared to be repressed.

The nonparental type of conidia purified from the fusant colony is most likely the result of diploidization, because the high frequency of recombinant colonies on the basis of uninucleate conidia of *Trichoderma* (*10,11*) cannot be explained. In addition, the ability of these conidia to segregate further confirms their diploid nature.

Exoglucanase production, although repressed to varying degrees in all the mutant haploids, was elevated to more than 100% of arithmetic mean of haploid parents in culture filtrates of both diploids A and B, suggesting the independent expression of the exoglucanase alleles. Intermediate production of endoglucanae and β-glucosidase by diploids indicates codominance of the alleles coding these enzymes or the dominance of the mutant allele. Absence of concomitant increase or decrease of cellulase component in both qualitative and quantitative assays reflects their independent control.

The segregants of both crosses revealed a range of enzyme titer of each component, indicating that they are polygenically controlled (*12*) Isolation of segregants with titers of glucoamylase and penicillin exceeding those of the better yielding parent has been reported (*5,12*). Thus, the potential of protoplast fusion techniques can be exploited for inducing parasexual recombination in industrially important imperfect fungi.

ACKNOWLEDGMENT

S. B. acknowledges the receipt of Junior Research Fellowship from the Council of Scientific and Industrial Research.

REFERENCES

1. Ogawa, K., Toyama, H., and Toyama, N. (1982), *J. Ferment. Technol.* **60,** 349–355.
2. Webster, J. (1964), *Sp. Trans. Br. Mycol. Soc.* **47,** 75–96.
3. Doi, Y. (1968), *Bull. Nat. Sci. Mus. Tokyo* **11,** 185–189.
4. Peberdy, J. F. (1980), *Enz. Microb. Technol.* **2,** 23–29.
5. Das, A. and Ghosh, A. (1989), *Biotechnol. Lett.* **2,** 705–708.
6. Bos, C. J. and Slakhorst, S. M. (1981), *Can. J. Microbiol.* **27,** 400–407.
7. Bos, C. J., Debets, A. J. M., Heusden, V. A. W., and Schepers, H. T. A. M. (1983), *Experientia (Suppl.)* **45,** 298–299.
8. Sandhu, D. K. and Kalra, M. K. (1982), *Trans. Br. Mycol. Sol.* **79,** 409–413.

9. Montenecourt, B. S. and Eveleigh, D. E. (1977), *Appl. Environ. Microbiol.* **33,** 178–183.
10. Rosen, D., Edelman, M., Gauln, E., and Dannon, E. (1974), *J. Gen. Microbiol.* **83,** 31–49.
11. Toyama, H., Yokayama, T., Shinmyo, A., and Okada, H. (1984), *Appl. Environ. Microbiol.* **47,** 363–368.
12. Bradshaw, R. E. and Peberdy, J. F. (1984), *Enzyme. Microb. Technol.* **6,** 121–126.