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# Production of Extracellular Enzymes in Mutants Isolated from *Trichoderma viride* Unable to Hydrolyze Cellulose

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Mutant strains not producing cellulases were induced and isolated from the cellulolytic fungus *Trichoderma viride*. Enrichment of mutants was carried out with the aid of nystatin selection. Mutants were shown to lack the ability to hydrolyze both soluble and crystalline cellulose. Mannanase and xylanase activities were also absent, indicating a common regulation for all these enzymes in *T. viride*. In some strains aryl-β-glucosidase activity was also missing. Mutants grew normally, but the amount of proteins secreted into the medium was very low, and in most cases these proteins were qualitatively different from the proteins of the parent strain.

Industrial-scale hydrolysis of insoluble cellulose-containing waste materials using cellulytic enzymes produced by microorganisms has become a common research interest throughout the world, as part of a search for potential sources of energy, food, and chemicals (4). Increased knowledge of the components and mode of action of the enzymes of the cellulase complex is required to facilitate the production of cellulases and selection of mutant strains suitable for use in industrial processes.

*Trichoderma viride* is a very efficient producer of cellulolytic enzymes, especially enzymes hydrolyzing crystalline cellulose (12). In this fungus cellulolytic enzymes form a synergistic complex of endoglucanases, which have been suggested to open free chain ends for exoglucanase to cleave off cellobiose units, which are broken down to glucose by β-glucosidase (1, 3, 20).

Cellulases are inducible enzymes, induced, for example, by cellulose and sophorose (10). Also, lactose has an inductive effect on cellulase production (14). In *Polyporus adustus* cellulose and glucomannan have been suggested to be inducers of the synthesis of cellulase, mannanase, and xylanase, probably acting through a common regulatory mechanism (5).

We have studied the regulation of this enzyme complex in the important cellulase-producing fungus *T. viride* by isolating strains not capable of hydrolyzing cellulose and determining whether the other enzymes were lost simultaneously. The results suggest that also in *T. viride* a common regulatory mechanism exists for this group of enzymes.

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## MATERIALS AND METHODS

**Strains and media.** *T. viride* QM 9414 (15), a good producer of cellulolytic enzymes, and the cellulase-negative mutant QM 9136 (15) were kindly donated by M. Mandels, Food Sciences Laboratory, U.S. Army Natick Laboratories, Natick, Mass. Strains were stored on potato dextrose agar slants.

Mutants were screened on plates containing minimum nutrient salts (14) and Walseth cellulose (23), using a modification of the method of Eriksson and Goodell (6). Instead of glucose, which represses the production of cellulases (11), 0.38% proteose peptone was used as an alternate carbon source for cellulase-negative mutants. By adding 0.1% Triton X-100 (Koch-Light Laboratories Ltd., Colnbrook, Bucks, England) into the medium, the size of the colonies could be kept reasonably small, about 5 to 10 mm in diameter after 5 days of incubation, without otherwise interfering with the morphology of the colonies.

The selection with nystatin was carried out on minimal plates using Avicel cellulose (type PH, Reinheitsgrad pharm., Serva Entwicklungslabor, Heidelberg) as carbon source. The nystatin overlay was prepared by mixing nystatin with potato dextrose agar.

Growth media for shake cultures were either as described by Mandels and Weber (14), using 1% Solka Floc BW 40 (Brown Co., Berlin, N.H.) as carbon source, or modified by replacing the Solka Floc with 0.4% lactose. All liquid media were buffered with 1.5% $KH_2PO_4$. Cultures were grown in 250-ml conical flasks containing 50 ml of medium. The cultivation temperature was 28°C, and the speed of the rotary shaker (New Brunswick MS2) was 200 rpm. After cultivation in shake flasks, the mycelium and possible solid particles remaining in the medium were centrifuged, and enzyme activities were assayed from the supernatant. To assay intracellular enzyme activity, mycelium was grown on lactose-containing medium for 3 or 6 days, collected by centrifugation, washed, and broken in an X-press, after which the enzyme activities were assayed from the suspension.

**Isolation of cellulase-negative mutants.** Mutants were induced from *T. viride* QM 9414 with either *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or gamma irradiation ($^{60}$Co). Conidial suspensions in 0.2 M sodium phosphate buffer, pH 7, were treated with the mutagenic agent. The final concentration of nitrosoguanidine (Fluka AG, Buchs SG, Switzerland) was 0.03%, and the treatment was carried out at 28°C for 1 to 3 h. The amount of irradiation given was 80 to 120 krads, using a dose rate of 100 krads/h.

Mutagenized conidia were then cloned on potato dextrose agar plates for 7 days. After cloning, cellulase-negative (*cel*) mutants were enriched using nystatin (Squibb & Sons, Inc., New York, N.Y.) as a selective agent (9), as described in more detail under Results. After selection surviving clones were screened on Walseth cellulose plates, and colonies not forming the clear transparent zone (cellulase-negative strains) in 10 days were selected for retesting on Walseth plates. Cellulase-negative colonies were then cultivated in shake flasks, and the enzyme activities described below were assayed from centrifuged growth media.

**Assays.** Activities against soluble cellulose, xylan, and mannan were assayed using the dinitrosalicylic acid method (21) by determining the reducing sugars formed from the substrates carboxymethylcellulose (Fluka), xylan (Fluka), and polygalactomannan (J. T. Baker Chemicals B.V., Deventer, Holland). Activity against crystalline cellulose was measured using the method of Leisola et al. (7). Aryl-β-glucosidase activity was assayed according to Norkrans (17) and β-galactosidase activity was assayed according to Pardee et al. (19).

Enzyme activity units were defined as follows: for soluble cellulose-hydrolyzing activity and xylanase, 1 unit is that amount of enzyme releasing 1 mg of reducing sugars per ml (in mannanase, 0.1 mg/ml) under assay conditions using glucose, xylose, or mannose, respectively, as the standard. For the measurement of crystalline cellulose-hydrolyzing activity, 1 unit gives an absorbance of 0.1 at 595 nm, when standard substrate is used. One unit of aryl-β-glucosidase releases 5 μmol of *p*-nitrophenol from the substrate (17), and 1 unit of β-galactosidase is calculated according to Pardee et al. (19).

Protein concentration was determined after trichloroacetic acid precipitation by the method of Lowry et al. (8), using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard.

**Growth measurement.** To test biomass production in liquid media, mycelia were harvested, dried, and weighed. Growth was indicated as milligrams (dry weight) of mycelia per 50 ml of culture medium.

**Immunological methods.** A dialyzed and lyophilized 4-day-old culture supernatant of *T. viride* QM 9414 grown on Solka Floc was used as immunogen. Antigens tested were also prepared in the same way from mutant strains.

Immunization was performed according to Wager and Räsänen (22), using white mice (BALB/c) received from the University of Turku. The technique used in immunodiffusion was that of Ouchterlony (18). The protein concentration of antigens was 0.5 to 1.0 mg/ml; antiserum was not diluted. A 10-μl amount of antigen was put into each well.

## RESULTS AND DISCUSSION

**Mutant isolation.** Cellulase-negative mutant strains were induced, enriched, and isolated from *T. viride* QM 9414. Regardless of the mutagenic agent used, the frequency of cellulase-negative mutants among survivors after the mutagen treatment was very low. For *P. adustus* it was about 0.02% (6). For *T. viride* in this work a comparable value of 0.08% was obtained without any selection. Mutant yields could be greatly increased using the nystatin enrichment technique (9). Selection was carried out on plates containing Avicel cellulose as the only carbon source. Thus, prototrophs able to grow on this carbon source were killed and *cel* mutants were enriched. An enrichment coefficient of cellulase-negative mutants of around 30 was obtained at the optimum concentration of nystatin (Fig. 1). The final percentage of cellulase-negative mutants among survivors of nystatin treatment was between 3 and 10%, which is within the range of enrichment percentages of auxotrophic mutants given in the literature (2). After selection, surviving colonies were screened on Walseth cellulose plates. Thirty cellulase-negative mutants not forming transparent zones around the colonies after two testings were selected for further characterization. All of these mutants were as sensitive to nystatin as was the parent strain QM 9414.

**Characterization of *cel* mutants.** Mutants not capable of hydrolyzing Walseth cellulose, the parent strain, and the *cel* control strain QM 9136 were cultivated in shake flasks using lactose or Solka Floc with proteose peptone as carbon sources. When lactose was used, biomass production by the different strains was measured. Proteose peptone sufficed for growth of the cel-

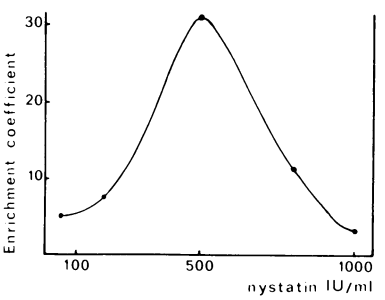


FIG. 1. *Effect of concentration (IU/ml) of nystatin on enrichment coefficient of cellulase-negative mutants. After 20 h of incubation on Avicel cellulose-containing minimal medium, different concentrations of nystatin mixed in potato dextrose agar were poured onto plates. The enrichment coefficient is the ratio of survival of a cel mutant (QM 9136) over the cel$^+$ parent (QM 9414).*

lulase-negative mutants. The growth of both the parent and *cel* mutants was quite similar using proteose peptone (0.075%) as sole carbon source, and it was almost as good as on lactose-containing medium.

After cultivation, extracellular enzyme activities were assayed from the culture supernatant. Results with typical mutants are shown in Table 1. These cellulase-negative mutants grown on Solka Floc medium seem to fall into two major classes. The first group consists of strains with extremely low cellulase, mannanase, xylanase, and aryl-β-glucosidase activities (*cel-1, cel-4, cel-7, cel-18, cel-24,* and *cel-17*). The strains in the other group (*cel-30, cel-22, cel-8,* and *cel-25*) have low cellulase, mannanase, and xylanase activities, but with aryl-β-glucosidase the activity is equal to or even greater than that of the parent, QM 9414. The mutant QM 9136, isolated by Mandels et al. (15), seems to belong to the former group.

Similar results were obtained when the strains were grown using lactose as the carbon source (Table 1). Generally the enzyme activities were about one-third of those obtained on the Solka Floc medium, except for mannanase activity in some mutants (*cel-7, cel-18, cel-24,* and *cel-17*), which was unexpectedly high. The growth of all mutants on lactose was similar to that of the parent strain (not shown).

All of the characterized mutants unable to hydrolyze Walseth cellulose had also lost the ability to secrete xylanase and mannanase. The observed simultaneous loss of these enzyme activities could be due to some general defect in enzyme secretion capability. However, this is probably not the case, because the secretion of β-galactosidase (Table 1), which does not belong to the cellulase complex, was quite normal or even increased in these mutants on both media, indicating that the *cel* mutation is more specific. The intracellular activity against soluble cellulose was also measured from some strains grown on lactose. No detectable activity was found in 6-day cultures of the mutants tested (*cel-1, cel-22,* QM 9136). In the parent QM 9414, at 3 days a fair amount (around 40%) of the activity was intracellular, and even at 6 days some activity (around 10%) was found in the mycelium.

An investigation was made of the growth characteristics and protein and enzyme secretion of two mutant strains, *cel-1* and *cel-22* (Fig. 2a and b, and 3a and b), representing the two major classes of cellulase-negative mutants. Solka Floc or lactose and proteose peptone were used as carbon sources. Strains QM 9136 (Fig. 4a and b) and QM 9414 (Fig. 5a and b) were used as controls.

Growth could only be measured from lactose media because solid particles of Solka Floc cellulose interfere with the biomass assay. Growth of the mutants was a little slower than that of the parent strain. When QM 9414 reached its highest peak, in about 4 days (Fig. 5b), mutants *cel-1* and *cel-22* (Fig. 2b and 3b) attained the same value in about 6 or 7 days. The maximum

TABLE 1. *Enzyme production of the parent strain and cel mutants in cultivations using Solka Floc (SF) or lactose (Lac) as the carbon source*[a]

| Strain | Soluble cellulose-hydrolyzing activity[b] | | Crystalline cellulose-hydrolyzing activity[c] | | Aryl-β-glucosidase activity[d] | | Mannanase activity[e] | | Xylanase activity[e] | | β-Galactosidase activity | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SF | Lac | SF | Lac | SF | Lac | SF | Lac | SF | Lac | SF | Lac |
| *cel-1* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.100 | 0.800 |
| *cel-4* | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 2 | 0 | 0.590 | 0.190 |
| *cel-7* | 0 | 0.5 | 0 | 0 | 0 | 0 | 0 | 35 | 8 | 5 | 0.050 | 0.060 |
| *cel-18* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 17 | 6 | 4 | 0.070 | 0.060 |
| *cel-24* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 16 | 0 | 2 | 0.100 | 0.260 |
| *cel-17* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 13 | 0 | 1.5 | 1.00 | 0.800 |
| *cel-30* | 10 | 0 | 6 | 0 | 0.100 | 0 | 12 | 0 | 20 | 0 | 0.910 | 0.040 |
| *cel-22* | 12 | 1.5 | 0.5 | 0 | 0.300 | 0.036 | 9 | 10 | 21 | 16 | 0.180 | 0.200 |
| *cel-8* | 8.5 | 0.6 | 2.5 | 2.5 | 0.193 | 0.087 | 16 | 0 | 17 | 0 | 4.12 | 0.330 |
| *cel-25* | 27 | 0 | 10 | 0 | 0.170 | 0.003 | 5 | 0 | 8 | 0 | 0.110 | 0.170 |
| QM 9136 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.060 | 0.170 |
| QM 9414 | 200 | 80 | 50 | 25 | 0.150 | 0.070 | 22 | 4 | 75 | 50 | 0.120 | 0.200 |

[a] Strains were grown for 7 days in shake cultures as described in Materials and Methods. Data are presented as enzyme activities in units.

[b] 0 = <0.01 unit.

[c] 0 = <0.2 unit.

[d] 0 = <0.001 unit.

[e] 0 = <0.2 unit.

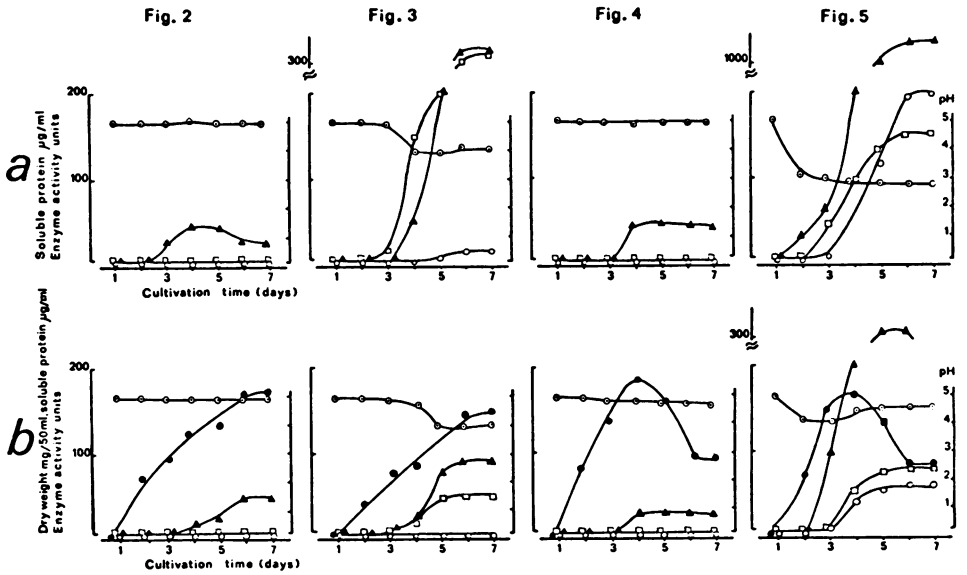


FIG. 2-5. *Growth and enzyme production of strains cel-1 (Fig. 2), cel-22 (Fig. 3), QM 9136 (Fig. 4), and QM 9414 (Fig. 5) on Solka Floc (a) and lactose (b) media. Growth (●), soluble protein (▲), carboxymethylcellulose-hydrolyzing activity (○), aryl-β-glucosidase activity (□), and pH (⊙) were followed for 7 days. Protein concentration is given in micrograms per milliliter, dry weight in milligrams per 50 ml, and enzyme activities in units (aryl-β-glucosidase units × $10^3$).*

values of growth were, however, very similar for all strains.

Strain *cel-1* (Fig. 2a and b) had no measurable aryl-β-glucosidase activity on either medium, nor had QM 9136 (Fig. 4a and b). Strain *cel-22* had high aryl-β-glucosidase activity on both Solka Floc and lactose media, with slight activities against soluble and crystalline cellulose on the Solka Floc medium (Fig. 3a and b). In *cel-22* production of aryl-β-glucosidase occurred at the same time as in the parent strain QM 9414 (Fig. 5a and b) on both media.

Mandels et al. (13) have shown that the amount of protein secreted into the growth medium correlates with cellulase activity. This correlation can also be seen in Fig. 2 to 5. In the mutants *cel-1* (Fig. 2a and b) and QM 9136 (Fig. 4a and b), there were no cellulolytic enzyme activities and also very little soluble protein. Strain *cel-22*, which had a high β-glucosidase activity, also produced high levels of protein. Excretion of protein seems to start when growth is incidentally decreased, reaching the greatest value when growth is already finished, as described earlier (13).

During cultivation, the changes in pH on Solka Floc medium correlated with production of cellulolytic enzymes (Fig. 2a to 5a). In mutants producing no cellulases, the pH remained at about the original level.

**Immunological test.** The proteins produced by mutants were studied immunologically to determine whether the mutants secreted the same proteins as the parent strain, but in inactive form or in much reduced amounts. Results are shown in Fig. 6.

No precipitation line was formed with any of the tested mutants, which lack both cellulase and aryl-β-glucosidase activities, showing that the proteins were quite different, since they had no cross-reacting material with the parent. Proteins of the mutant *cel-22* having a high aryl-β-glucosidase activity did, however, show a precipitation reaction, probably due to the aryl-β-glucosidase produced.

**Revertants.** To determine whether one or more genes had mutated, revertants were induced with nitrosoguanidine from both types of cellulase-negative mutants and screened on Walseth plates. No revertant colonies were found from the type with no measurable cellulase or aryl-β-glucosidase activities. Mandels et al. (15) were also unable to find any revertants from the cellulase-negative mutant QM 9136. However, revertants were found, with low frequency, from the other mutant type with low cellulase and high aryl-β-glucosidase activities. All enzyme activities studied in revertants were approximately at the parent strain level. This fact indicates that only one gene was involved in the original mutation. In the mutant class in which aryl-β-glucosidase was also lost, the lack of re-

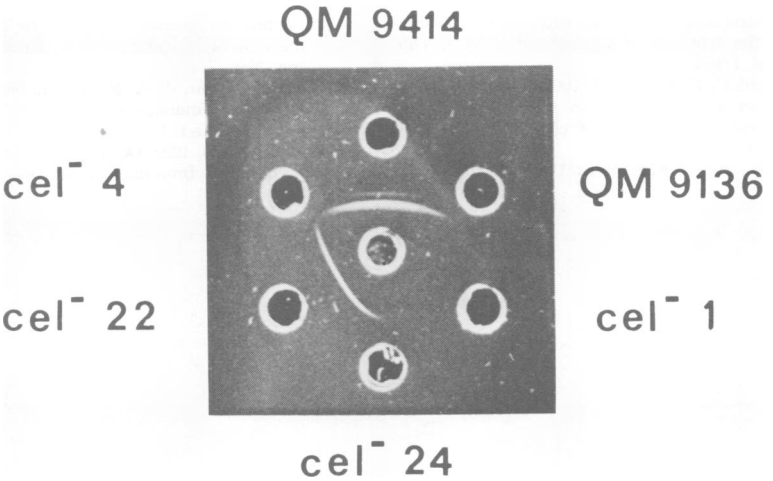


FIG. 6. *Immunological testing of extracellular proteins of mutant strains. Antiserum produced against the proteins of QM 9414 was placed in the center well. The surrounding wells contain 10 μl of protein, at a concentration of 1 mg/ml, from strains QM 9414, QM 9136, cel-1 (cel⁻ 1, etc., on figure), cel-22, cel-4, and cel-24.*

vertants suggests that they are multiple mutants or perhaps deletions lacking the structural genes for these enzymes.

These results, showing that a single-step reversible mutation can lead to concomitant loss of crystalline and soluble cellulose-hydrolyzing cellulase, xylanase, and mannanase activity without affecting β-galactosidase, suggest that the mutation is involved in a common regulatory mechanism for the production of all these proteins. Aryl-β-glucosidase seems to be separately regulated, as is the case also in *P. adustus* (6) and *Neurospora crassa* (16).

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