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Induction of Mutation in *Trichoderma viride* for Conversion of Natural Cellulose into Glucose

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INTRODUCTION

The production of cellulolytic enzymes from fungi has been extensively studied. Several mutants of *Trichoderma reesei* were selected (*1*). Most of the studies were carried out on *T. reesei* (*2,3*), *T. viride* (*4–6*), *T. harzianum* (*7*), *Penicillium funiculosum* (*8*), *Alternaria alternata* (*9*). *Aspergillus phoenicis* (*10*), *A. ustus* (*11*), *A. tamarii* (*12*), *A. japonicus* (*13*), and *A. niger* (*14*). *T. koningii* is one of the most active producers of the so-called C_1 factor, which is indispensable for the rapid and extensive attack on crystalline cellulose (*15*). However, *Trichoderma* is known to excrete only small amounts of β-glucosidase (*16*). Therefore, *Trichoderma* is supplemented with β-glucosidase from *Aspergillus* to increase the saccharification rate of cellulose to glucose as the main sugar (*3*). Induction of mutations in *Trichoderma* spp. rather than *T. viride* as a tool for the enhancement of β-glucosidase activity was reported. Unfortunately, *T. reesei* is a poor producer of β-glucosidase. On the other hand, *T. harzianum* M_5, a mutant that was induced by gamma radiation (*17*), produced high yields, not only of Avicelase and carboxy methyl cellulase, but also of β-glucosidase, than its respective wild type.

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The present work was carried out aiming to select some mutants of *T. viride* with higher efficiency to produce different cellulases, especially β-glucosidase, than its parent strain.

MATERIALS AND METHODS

Microorganisms

Trichoderma viride ATCC-32630 was obtained from the American Type Culture Collection, MD. The fungus was grown at 25°C for 7 d on potato dextrose agar slants (*18*) and maintained on the same medium at 4°C. Subculturing was carried out at monthly intervals.

Growth and Growth Media

The minimal medium was prepared as previously described (*19*). The liquid-growth medium was prepared as described by Duff et al. (*3*).

Induction and Selection of Mutants

Mutation was enhanced by UV-irradiation. A suspension of 10^7 spores mL^{-1} was poured into glass Petri dish (10 cm diameter) and subjected to UV-source (2400 Erg/mm^2) under stirring at different intervals.

Assay of Cellulolytic Enzymes Activity

The assay of Avicelase, CMCase, and β-glucosidase activity was carried out as described by Helmi et al. (*20*).

On laboratory scale, the saccharification of cellulose powder and rice straw was carried out using crude enzyme preparations of *A. niger*, *T. viride*, and a mixture of crude enzyme preparations of both. The hydrolysis mixture contained 2 mL of crude enzyme preparation, 2 mL of 0.1 *M* acetate buffer, pH 4, and 200 mg of cellulosic substrate. The reaction mixtures in 20 mL screw capped test tubes were incubated for 4 h at 50°C with constant rotary shaking at 200 rpm. Assay of Avicelase, CMCase, β-glucosidase, and total reducing sugars as glucose equivalent was carried out as described above.

RESULTS

The effect of UV-irradiation on a conidia suspension of *T. viride* have indicated that an exposure time of 60 s was found to be appropriate since about 5% survivors could be obtained. Among 5000 colonies, 100 colonies, were selected on the basis of rapid growth and early conidiation on solid medium containing 1% cellulose powder or Avicel (microcrystalline cellulose) as the sole source of carbon, after 7 d at 25°C. The time needed for

Table 1
Cellulolytic Enzymes Activities of the Wild Type and Selected Mutants of *Trichoderma viride* after Cultivation in Liquid-Medium at 28°C for 12 d under Shaking (200 rpm)

Mutant	Cellulolytic enzyme activities		
	Avicelase (μM reducing sugars/ml/min)	CMCase (μM reducing sugars/ml/min)	β-Glucosidase (μM p-nitro-phenol/ml/min)
Wild type	0.0330	3.76	21.00
M1	0.0605	4.58	15.33
M2	0.0165	4.05	17.55
M3	0.0475	3.82	19.22
M4	0.0335	3.56	14.77
M5	0.0375	4.91	29.95
M6	0.0305	4.35	16.72
M7	0.0073	0.47	6.69
M8	0.0200	1.36	9.20
M9	0.0115	0.85	9.83
M10	0.0115	0.77	10.45
M11	0.0125	0.92	10.03
M12	0.0270	1.44	16.72
M13	0.0295	4.61	12.89
M14	0.0385	5.74	12.54
M15	0.0215	2.74	6.27
M16	0.0565	3.53	19.51
M17	0.0210	2.59	20.20
M18	0.0080	1.24	3.49
M19	0.0365	1.82	17.42
M20	0.0285	2.53	18.11

colonization and conidiation varied between 34–42 h and 72–92 h, respectively. The selected colonies were resubcultured on a minimal medium and examined after 7 d of incubation at 25°C for morphological differences. Two types of colonies could be identified, one was green in color and the other was green with yellow center; however, such difference disappeared by growing both on potato-dextrose agar medium. Ten mutants of each color type were selected for further investigations regarding the activity of different cellulases, including Avicelase, CMCase, and β-glucosidase.

The data in Table 1 indicates variations in the activity values of the 20 selected mutants. Some of these showed activity values higher than that detected for the wild strain, whereas others revealed lower activity. Six of

the selected mutants revealed higher Avicelase activity; 0.065, 0.0475, 0.0375, 0.0385, 0.0565, and 0.0365 μM reducing sugars for the mutants M1, M3, M5, M14, M16, and M19 than their respective wild type, 0.033 μM reducing sugars/mL/min. UV-irradiation of *T. viride* induced also some mutants that exhibited higher CMCase activity than the wild type. The mutants M1, M5, M6, M13, and M14 indicated CMCase activity values of 4.58, 4.91, 4.35, 4.61, and 5.74 μM reducing sugars/mL/min, respectively, compared with the wild type, which revealed an activity value of 3.76 μM reducing sugars/mL/min. On the other hand, only one mutant designated as M5 showed increased activity toward the hydrolysis of cellobiose. That mutant revealed β-glucosidase activity of 29.95 μM *p*-nitrophenol/mL/min, which is higher than the activity of the wild type, 21.0 μM *p*-nitrophenol/mL/min.

On laboratory scale, the saccharification of either cellulose powder as a model substrate and one of natural cellulosic materials, namely rice straw, by crude enzyme preparations of *A. niger, T. viride,* and mixture of both indicated the superiority of *T. viride* enzyme system than the crude enzyme preparation of the mold *A. niger* (Table 2). The values obtained for the crude enzyme preparation of *T. viride* were 0.0125 and 0.0133 μM reducing sugars/mL/min for cellulose powder and rice straw, respectively, whereas the saccharification rate for the above substrates was found to be 0.0033 and 0.0039 μM reducing sugars/mL/min when the crude enzyme preparation of *A. niger* was used. The saccharification rate of cellulose powder and rice straw by a mixture of the culture filtrate of the experimented fungi species was much better increased than on using the crude enzyme preparation of *A. niger* alone.

DISCUSSION

Studies concerning growth and cellulases production by *T. viride* indicated that the highest mycelial weight could be obtained after 10 d of incubation at 28°C, whereas maximum activity was detected after 12 d for the Avicelase, and 14 d for the CMCase. It seems that growth rate and production of cellulases may vary from one species to another. *T. harzianum* revealed maximum activity of different cellulases after 6 d of incubation (*17*), whereas *T. koningii* showed the highest capacity to secrete cellulases after 8 d (*21*).

It is known that *T. viride* secretes small amounts of β-glucosidase (*16*), therefore, it was tried before (*10*) to supplement *Trichoderma* cellulases with β-glucosidase preparation from *Aspergillus.* The second approach was to raise the β-glucosidase activity of *T. viride* by UV-irradiation, as mutant 5 obtained during the present investigation revealed higher activity of such enzyme (30 μM *p*-nitrophenol/mL/min) than its respective wild strain (21 μM *p*-nitrophenol/mL/min). However, such increase is very much lower than that reported for the wild types of other fungi species (*9,17,20*).

Table 2
Saccharification Rate of Cellulose Powder and Rice Straw by the Crude Cellulolytic Enzymes Prepared from *Aspergillus niger*, *Trichoderma viride*, and their Mixture

Source of culture filtrate*	Concentration of protein (mg/ml)	Cellulolytic enzymes activities			Saccharification rate (μM reducing sugars/ml/min)	
		Avicelase (μM reducing sugars/ml/min)	CMCase	β-Glucosidase (μM p-nitrophenol/ml/min)	Cellulose powder	Rice straw
Aspergillus niger	0.71	0.0105	4.36	142.09	0.0033	0.0039
Trichoderma viride	0.57	0.0450	3.08	14.63	0.0125	0.0133
Mixture** of A. niger and T. viride	0.64	0.0125	3.97	80.79	0.0102	0.0097

* After 8- and 12-days cultivation for Aspergillus niger and Trichoderma viride, respectively.
** At the ratio of 1:1.
The values indicated in the table are the mean of two replicates and four determinations.

On minilaboratory scale, the increased saccharification rate of cellulose powder and rice straw on using *T. viride* enzymes extract than that of *A. niger* could be explained by the higher Avicelase activity of the former than that of the latter. Further investigation has indicated that a mixture of enzyme extracts of *T. viride* and *A. niger* is better for the saccharification of the two substrates used than on using the crude cellulases of *A. niger*. Previous studies (22) confirmed such trend on using mixture of cellulases of different fungi species.

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