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# Induction of Mutation in *Aspergillus niger* for Conversion of Cellulose into Glucose

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## INTRODUCTION

Plant wastes are very important part of biomass used and investigated for energy, chemical, and fuel production (*1*). Cellulose is the major renewable form of carbohydrate in the world, about $10^{11}$ tons of which is synthesized annually. For general use, it must be hydrolyzed first, either chemically or by cellulases derived from a few specialized microorganisms (*2*). Enzymes are acceptable environmentally but expensive to produce. Certainly, induction of mutations and selection of high cellulase microbial strains with significant adaptability to degrade cellulose to glucose is promising solutions. Induction of mutations in other fungi and *Aspergillus* sp. rather than *Aspergillus niger* was reported. *Aspergillus ustus* and *Trichoderma harzianum* were induced by gamma irradiation indicating mutants that excrete higher cellulase yields, particularly exocellobiohydrolase (Avicelase) than their respective wild types (3). Mutants from the cellulolytic fungus *Penicillium pinophilum* were induced by chemical and UV-irradiation (4). Enhancing the production of endo-1,4-β-D-glucanase (CMCase) and particularly β-glucosidase was obtained by gamma irradiation of *Alternaria alternata* (*5*). To overcome the lower activity of β-glucosidase in certain fungi species rather than *A. niger,* mixed cultures of different species were tried. Thus, *Aspergillus phonicis* with *Trichoderma reesei* Rut 30, produced a cellulase complex that improved activity twofold over cellulase from *Trichoderma* alone (*6*).

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The present work deals with the effect of UV-irradiation on the possibility of inducing some mutants of *A. niger*, that excretes higher activity of different cellulases than their respective wild type, since nothing was reported in the literature about the enhancement of cellulases activity by inducing mutations in the above fungi species.

## MATERIALS AND METHODS

### Microorganism

*A. niger* ATCC-1004 used in the present study was supplied by the American Type Culture Collection, MD. The fungus was grown at 27°C on potato-dextrose agar slants (*7*) and maintained on the same medium at 4°C. Subculturing was carried out at monthly intervals.

### Growth and Cultivation Media

The minimal medium was prepared as described (*8*), and contained $gL^{-1}$: sodium nitrate, 6; magnesium sulfate·$7H_2O$, 0.52; potassium chloride, 0.52; few crystals of ferrous sulfate·$7H_2O$; zinc sulfate·$7H_2O$; copper sulfate·$5H_2O$, and glucose, 10. The pH was adjusted to 6.3 and 1.5% agar was added and sterilization carried out at 121°C for 15 min.

The liquid-growth medium was prepared as (*3*) and contained $gL^{-1}$: sodium dihydrogen phosphate, 12; potassium dihydrogen phosphate, 2; diammonium hydrogen *o*-phosphate, 7; magnesium sulfate·$7H_2O$, 0.3; calcium chloride·$2H_2O$, 0.3; urea, 0.3; proteose peptone, 0.25; yeast extract, 0.1; Tween-80, 0.3; ferrous sulfate·$7H_2O$, 5 mg; manganese sulfate·$7H_2O$, 15 mg; zinc sulfate·$7H_2O$, 34 mg; cobalt chloride·$6H_2O$, 4 mg and D-glucose, 10. Before sterilization at 121°C for 15 min, the medium pH was adjusted to 5 with 0.1 *M* $H_3PO_4$.

*Aspergillus* selective solid-growth medium was the same as the *Aspergillus* liquid-growth medium except that 1% (w/v) of cellulose powder or Avicel instead of glucose as a source of carbon and 2% agar were added.

### Induction and Selection of Mutants

A 5 mL of $10^6$ conidia suspension were exposed to UV (2400 Erg/$mm^2$) at different intervals. An exposure time of 120 s was found appropriate as 95% of the spores were killed and the growing colonies were replated on the solid-growth medium supplemented with 1% (w/v) cellulose powder or Avicel (selective medium). Mutants were selected on the basis of rapid growth and early conidiation. Further selection based on Avicelase, CMCase, and β-glucosidase activity was carried out by growing the selected mutants in liquid-growth medium supplemented with 1% (w/v) cellulose powder (dry-oven-sterilized at 120°C for 30 min) after 30 h of incubation.

## Characterization Studies

*Colonization*

Suitable dilutions of spore suspensions were plated on potato-dextrose agar medium and incubated at 27°C for 7 d. The time of colonization was recorded.

*Germination*

The spores were suspended in 5.0 mL of sterile liquid potato dextrose medium and incubated at 27°C for 6, 8, and 10 h in aquatherm water bath shaker (200 rpm). The germinated conidia were examined under light-microscope and the percentage of germination was calculated.

## Assay of Cellulolytic Enzymes Activity

The assay of Avicelase (exocellobiohydrolase) and CMCase (endo-1,4-β-D-glucanase) was carried out by measuring the reducing sugars by the Somogyi (*9*) and Nelson (*10*) method as described by Hong et al. (*11*), with some modifications. The activity of β-D-glucosidase was measured by using *p*-nitrophenyl β-D-glucoside (pNPG) as described by Herr (*12*).

## RESULTS

The rate of growth of some selected mutants as determined from the time of colonization in comparing with the wild type is shown in Table 1. The data indicated shifts in the colonization time of the mutants as a result of irradiation, as well as difference between the selected colonies in that respect. Such difference could be seen between mutant No. 11, which revealed colonization after 40 h, and mutant No. 7, which started to form colonies after 20 h, whereas the wild type showed colonies after 24 h.

As shown in Table 2, a sharp difference in the percentage of germination between the wild type (75%) and some selected mutants was detected after the same time of incubation. Mutant No. 8 (6.7% germination after 10 h incubation), whereas mutant No. 6 (75% after 10 h) on the other hand showed a similar percentage of germination as that detected for the wild type, even though it did not germinate at all after 6 h of incubation.

Figure 1 shows the Avicelase activity of the mutants investigated. Only three mutants revealed higher activity as 0.0205, 0.0183, and 0.0295 $\mu M$ reducing sugars/mL/min for $M_2$, $M_7$, and $M_8$, respectively than 0.0153 $\mu M$ reducing sugars/mL/min for the wild type. The same trend was observed for the carboxymethylcellulase activity as some of the mutants secreted higher activity of the CMCase than the wild type (Fig. 2). The mutants designated $M_2$, $M_3$, $M_5$, $M_7$, and $M_8$ revealed CMCase activity in the order of 6.47, 5.30, 3.06, 7.20, and 3.44 $\mu M$ reducing sugars/mL/min, which are higher than that detected for the wild type, 2.94 $\mu M$ reducing sugars/mL/

Table 1
Colonization Time of the Wild Type and the Eleven Selected Mutants after Plating on Potato-Dextrose Agar Medium at 27°C

| Mutant | Colonization time (h) |
|---|---|
| Wild type | 24 |
| 1 | 22 |
| 2 | 22 |
| 3 | 24 |
| 4 | 24 |
| 5 | 22 |
| 6 | 22 |
| 7 | 20 |
| 8 | 28 |
| 9 | 22 |
| 10 | 25 |
| 11 | 40 |

min. Also, Fig. 3 indicates that five of these mutants designated $M_1$, $M_2$, $M_3$, $M_5$, and $M_7$ gave higher $\beta$-glucosidase activity values, 267.5, 255, 255.7, 196.5, and 255 $\mu M$ *p*-nitrophenol/mL/min, respectively, as compared to the wild type, 167.2 $\mu M$ *p*-nitrophenol/mL/min.

The above figures confirm the stability of some of the mutants toward the synthesis of different cellulases as they showed increases in the activity of the enzymes, or even stability by growing in liquid medium for different intervals.

## DISCUSSION

To reduce the number of mutants to be tested for possible changes in cellulolytic enzymes activity of UV-irradiated conidia of *A. niger*, a preliminary course of selection was followed. During the course of selection, the rate and capacity of the irradiated condidia to grow (colonization) on potato-dextrose agar medium was examined. Among 5000 growing colonies inspected, only 11 mutants were selected on the basis of the time needed for colonization as compared to the wild type, which showed a colonization time of 24 h, whereas those that showed more than 40 h were omitted. In addition to the colonization behavior of the growing colonies,

Table 2
The Rate and Percentage of Germination of conidia
of the Wild Type and the Eleven Selected Mutants*

| Mutant | Germination (%) Incubation time (hours) | | |
|---|---|---|---|
| | 6 | 8 | 10 |
| Wild type | 21.50 | 45.90 | 75.00 |
| 1 | 26.35 | 55.50 | 75.00 |
| 2 | 30.00 | 37.50 | 45.45 |
| 3 | 27.30 | 45.45 | 50.00 |
| 4 | 7.75 | 40.00 | 46.70 |
| 5 | 46.13 | 58.40 | 66.70 |
| 6 | 0.00 | 57.10 | 75.00 |
| 7 | 57.10 | 66.70 | 68.80 |
| 8 | 0.00 | 00.00 | 6.70 |
| 9 | 42.90 | 68.20 | 74.00 |
| 10 | 57.10 | 80.00 | 88.50 |
| 11 | 0.00 | 66.70 | 70.00 |

*Incubation was carried out at 27°C using potato-dextrose liquid medium for different times.

$$\text{Germination \%} = \frac{\text{Number of germinated conidia}}{\text{Total number of conidia}} \times 100$$

the pattern of germination of conidia from the selected colonies and its percentage were considered also as base for preliminary selection. A sharp difference in the time as well as in the percentage of germination between the wild type and the previously irradiated and subcultured conidia was found. On that base, the previously selected colonies gave good germination behavior compared to the wild type and were used for subsequent investigations for probable changes in cellulases activity. On the other hand, the mutants that showed long or even no capacity to germinate were neglected.

The mold *A. niger* (wild type) and its mutants induced by UV irradiation revealed higher carboxy methyl cellulase and very much higher activity values of β-glucosidase than *A. ustus* $M_{35}$, *Alternaria alternata* $M_7$ (*13*), and *A. japonicus* (*14*). Thus, it can be said that *A. niger* could be considered as the most appropriate fungi species with respect to the activity of β-glucosidase that is actually responsible for the saccharification of cellulose. Previous studies have indicated that *Aspergillus* sp. are suitable

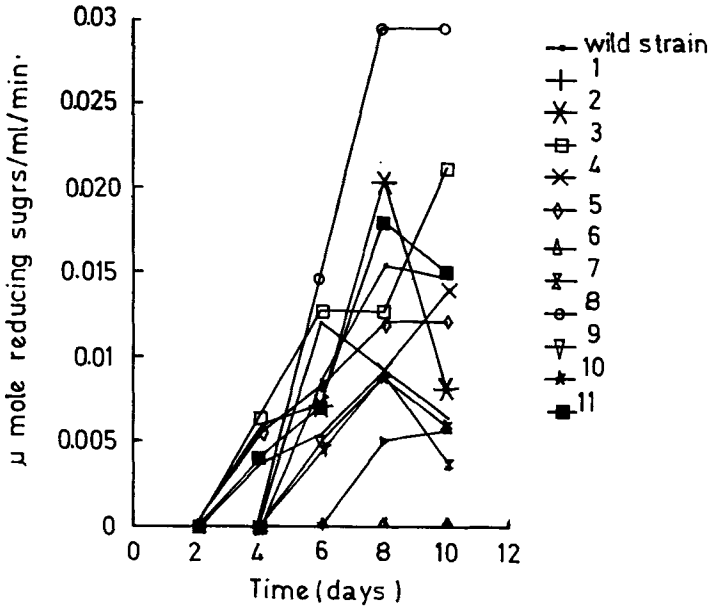


Fig. 1. Effect of the time of cultivation of *A. niger* (wild strain) and its mutants on the Avicelase activity.

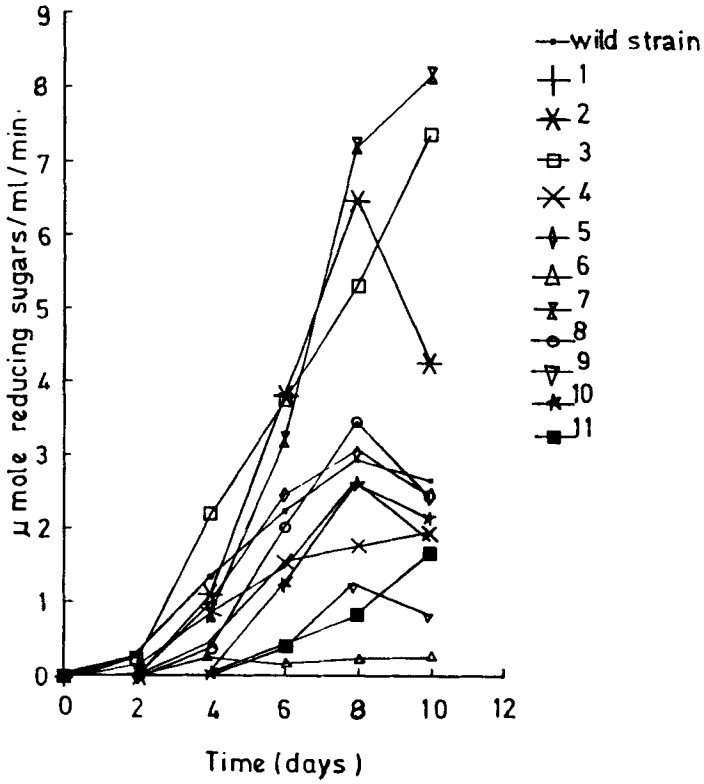


Fig. 2. Effect of the time of cultivation of *A. niger* (wild strain) and its mutants on CMCase activity.

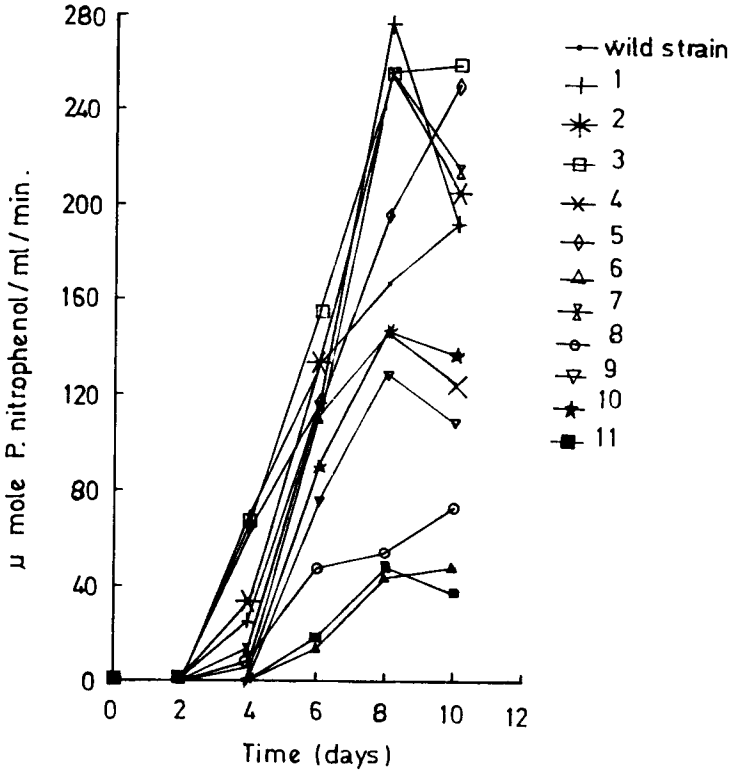


Fig. 3. Effect of the time of cultivation of *A. niger* (wild strain) and its mutants on the β-glucosidase activity.

for the production of β-glucosidase. Furthermore, the enzyme is less sensitive to end product inhibition than that of *Trichoderma reesei* (*6*). Therefore, *Trichoderma* cellulases were supplemented with β-glucosidase from *Aspergillus,* and this caused increased rate of saccharification of cellulase to glucose as the main sugar (*15*).

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