0273-2289/93/3940—0289$03.00

Intensification of Glucose Oxidase Synthesis by Multistage Mutagenesis of *Aspergillus niger*

Scientific Note

M. K. TAHOUN

Department of Agricultural Industries, Faculty of Agriculture, University of Alexandria, Egypt

INTRODUCTION

Glucose oxidase (E.C. 1.1.3.4) has many applications either clinically for the determination of glucose in body fluids (e.g., blood and urine) (*1*) or in food industries (*2*). The use of *Aspergillus niger* as a source of the enzyme glucose oxidase was reported (*3,4*).

Mutagenic activation of microbial cultures was used for enhancing the synthesis of industrial enzymes (*5,6*). On treating conidia of *A. niger* G with UV and nitrosoguanidine (NTG), glucose oxidase activity could be increased by 1.5–1.8% (*7*). Although recent investigations have led to the cloning of glucose oxidase gene (*8,9*), Swart et al. (*10*) reported the characterization of the nine *gox* loci on chromosomal DNA of *A. niger*, indicating a series of genes affecting its glucose oxidase level.

This article describes a modification for the screening of *A. niger* glucose oxidase productivity, as well as intensification of enzyme synthesis by the way of multistage mutagenesis.

MATERIALS AND METHODS

Aspergillus niger 8/17 was supplied by the Department of Botany, University of Nottingham, Nottingham, UK. The fungus was maintained on malt yeast glucose agar (MYG) at 4°C. Conidia were washed by suspension in 0.01% Tween 80 and centrifugation. The process was repeated several times for the removal of an associated dark pigment.

Chemicals

Acryflavin (a mixture of 2,6-diamino-10-methyl acridinium chloride and 2,8-diamino acridine) and 5-fluorouracil (2,4-Dihydroxy-5-fluoroprymidine) were obtained from Sigma Chemical Company, St. Louis, MO. Imazalil (1-β-[alkyloxy]-2,4-chloro phenetyl-imidazole phosphate) was a gift from J. F. Peberdy, Department of Botany, University of Nottingham, Nottingham, UK. Acryflavin and 5-fluorouracil were dissolved in distilled water, whereas imazalil was dissolved in dimethyl sulfoxide and added to molten agar to give a maximum solvent concentration of 1%. All other chemicals were from B. D. H. Poole, Dorest, UK.

Media

The MYG agar contained (g/L): malt extract (Oxoid), 5.0; yeast extract (Oxoid), 5.0; D-glucose, 2.5; agar, 20.0; and $CuSO_4 \cdot 5H_2O$, 0.001.

The agar medium used for the screening of mutated conidia consisted of two layers: The lower one (10 mL, 2% agar) contained (g/L): $(NH_4)_2HPO_4$, 0.338; KH_2PO_4, 0.188; $MgSO_4 \cdot 7H_2O$, 0.156; $CaCO_3$, 35.0 in 0.1*M* McIlivin buffer pH 5.6. The upper layer (8 mL, 2% agar) contained (g/L): D-glucose, 80.0; soluble starch, 10.0; sodium deoxycholate, 0.2; and potassium iodide, 0.34 in 0.1*M* McIlvin buffer, pH 5.6.

The mutants that showed the greatest diffusion areas on the diagnostic medium were further examined for enzyme secretion by cultivation in liquid medium that contained (g/L): D-glucose, 80.0; peptone (Sigma), 3.0; $(NH_4)_2HPO_4$, 0.388; KH_2PO_4, 0.188; $MgSO_4 \cdot 7H_2O$, 0.156; and $CaCO_3$, 35.0 (sterilized separately). Fifty milliliters of the above medium were transferred into 250-mL Erlenmeyer flasks and sterilized at 110°C for 30 min. The flasks were inoculated with 0.5 mL of a 10^7 spores/mL spore suspension and incubated at 30°C with shaking (200 rpm) for 72 h.

Determination of Glucose Oxidase Activity

Glucose oxidase activity was assayed in culture fluids by a modification of Lloyd and Whelan method (*11*). To 100 μL of the culture supernatants, 2.0 mL of a reaction mixture prepared by dissolving 100.0 mg of D-glucose, 5.0 mg of *O*-dianisidine (Sigma), and 3.0 mg of peroxidase (Sigma) in 100 mL of 0.3*M* tris-phosphate-glycerol buffer, pH 7.5, were added. The reaction mixture was incubated at 30°C for 30 min, and 2 mL of 5*M* HCl were added. The developed red color was measured at 525 nm against a blank. One enzyme unit (U) is defined as that amount of enzyme that produces 1 μmol of hydrogen peroxide or glucose/mL of the supernatant culture fluid in 30 min.

Mutagenesis

The induction of mutation in *Aspergillus niger* 8/17 was stimulated by exposing a conidia suspension of 7-d-old cultures to UV irradiation. Five milliliters of conidia suspension (10^7 spores/mL) were exposed to UV source 2400 erg mm^2 in a 4.5-cm open plastic Petri dish for different intervals. Counting of viable spores was carried out on MYG after 3 d of incubation at 28°C.

Induction of mutation in *A. niger* by antifungal inhibitors and 5-fluorouracil was carried out by determining the minimum inhibitory concentration (MIC) of each (defined as the minimum concentration that prevents germination of conidia and growth) at which mutants will be selected. Thus, a heavy conidia suspension was laid on solidified MYG previously mixed with 5-fluorouracil, acryflavin, or imazalil at three levels of concentration (1–10, 10–100, and 100–1000 μg/mL), and the plates were incubated at 28°C for 3 d.

The effect of UV and three times the MIC of imazalil on the induction of mutants with glucose oxidase activity exceeding the parent *A. niger* 8/17 was investigated. A conidia suspension (10^7 spores/mL) was irradiated first with UV to give 30% survivors. One milliliter of the mutated conidia was laid over MYG for 10 h (expression time), followed by a second layer of MYG (~7 mL) containing 6 μg/mL of imazalil, and incubation of plates was carried out for 3 d at 28°C.

RESULTS AND DISCUSSION

The influence of UV irradiation at 2400 erg mm^2 indicated the interference of a dark pigment that was associated with conidia giving nonreproducible percent survivors. Washing conidia with 0.01% Tween 80 several times gave better results, as shown in Fig. 1.

The use of the diagnostic medium for preliminary selection of mutated *Aspergillus niger* conidia as indicated previously (*7*) did not permit the germination of conidia. It was found that decreasing the concentration of potassium iodide to 0.34 g/L significantly improved the screening process.

On determining the MIC of different antifungal inhibitors, it was found that for imazalil to be at 2 μg/mL, whereas the MIC for acryflavine and 5-fluorouracil was detected at concentrations of 700 and 1000 μg/mL, respectively. Accordingly, it is not recommended to select mutants at the MIC of either acryflavine or 5-fluorouracil, since it was stated that high dose of mutagen could result in chromosomal aberrations (*12*) and in general disturb the genetic background by an enhanced load of undesirable mutations, especially when recurrent mutagenic treatment is given, as is often the case with strains of industrial interest.

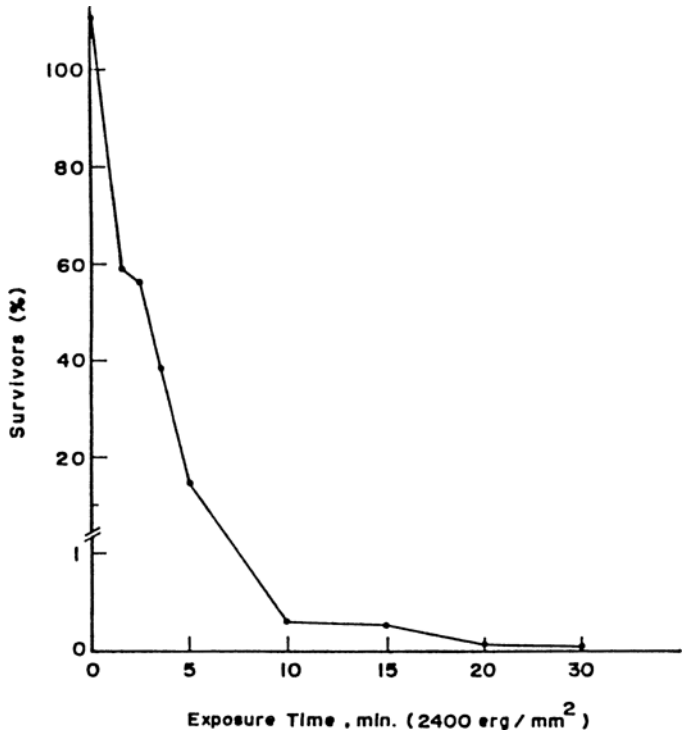

Fig. 1. Survivors of *A. niger* 8/17 after exposure to UV 2400 erg mm² for different intervals (min).

Induction of mutation in *A. niger* 8/17 by simultaneous treatment of conidia suspension by UV irradiation was carried out on two stages. In the first stage, the conidia (10^7 spores/mL) were UV irradiated at 2400 erg mm² to give 8% survivors, followed by plating on MYG at a density that permits the possibility of single-spore growing colonies. For screening the mutants with respect to their efficiency in glucose oxidase productivity, about 1000 colonies were replated on the diagnostic medium. On the basis of the magnitude of enzymatic zone around the growing colonies, 25 colonies were selected for further investigation for their capacity to produce glucose oxidase in liquid culture medium. Of 25 mutants tested, 21 showed higher glucose oxidase activity than the parent strain of *A. niger* 8/17 (9.57 U/mL). The activity of the 25 mutants varied between 9.41 and 22.94 U/mL with a mean value of 12.5 U/mL as shown in Fig. 2, which was significantly higher than that of the parent strain 8/17 ($t_{24}=2.06$, $p>0.05$). The mutant that showed the highest glucose oxidase activity (22.94 U/mL) was remutated by UV irradiation to give 7% survivors. Among the 25 mutants selected out of 1000 growing colonies, only one mutant revealed higher glucose oxidase activity (25 U/mL, Fig. 3). The mean value of glucose oxidase of the tested mutants (13.52 U/mL) was

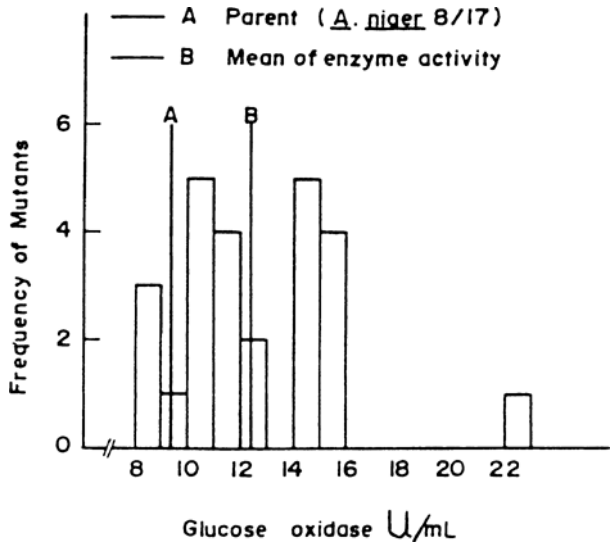

Fig. 2. Glucose oxidase activity U/mL of *A. niger* 8/17 mutants obtained during the first step of UV irradiation.

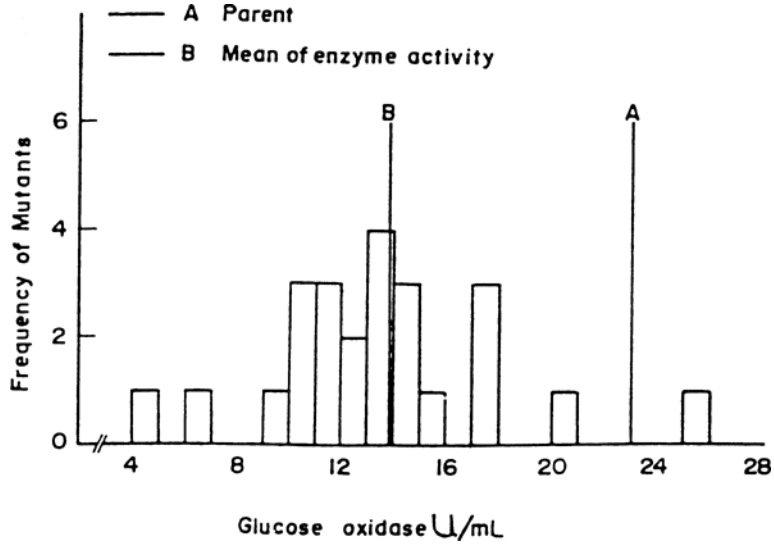

Fig. 3. Glucose oxidase activity U/mL of mutants of *A. niger* obtained by UV irradiation of previously UV-treated conidia.

significantly lower than that of the starting strain ($t_{24}=2.06$, $p>0.05$). The above data indicate less damage of chromosomal DNA during the first stage of UV treatment, since only four mutants out the 25 mutants selected for glucose oxidase activity showed lower enzyme activity than the starting strain 8/17. The increase in glucose oxidase activity as a result of multistage mutagenization of *A. niger* 8/17 on two stages reached 164%,

Table 1
Glucose Oxidase Activity of Color Mutants Isolated from *A. niger* 8/17 by UV and Imazalil Mutagenization

Strain	Glucose oxidase activity U/mL/30 min, μmol glucose	Glucose oxidase activity, %	Increase of glucose oxidase activity, %
A. niger 8/17	1.55	100.00	-
Color mutant			
White	1.77	114.54	14.54
Black	2.88	186.36	85.36
Dark brown	3.05	197.37	97.37
Brown	3.11	201.25	101.25
Gray	3.22	208.37	108.37
Green	3.66	236.84	136.84

which is higher than fivefold the increase reported for *A. niger* G13 mutated by the way of multistage mutagenization using different combinations of UV plus nitroso methylurea, ethyleneimine, acryflavine, or *N*-methyl-*N*-nitrosoguanidine (*13*).

The influence of the combination of UV irradiation and imazalil on the induction of mutation in *A. niger* 8/17 gave rise to some color mutants. Table 1 indiates six mutants with glucose oxidase activity greater than that of the parent. That increase varied between 14.54 and 136.84 U/mL, which is similar to the results obtained during the first stage of mutagenization by UV at 2400 erg mm^2. The increase in glucose oxidase activity by the methods used in the present study was much higher than those reported previously (*7,13*).

ACKNOWLEDGMENTS

The research was supported partially by a British Council Scholarship in the UK. I thank Professor J. F. Peberdy, and Department of Botany, the University of Nottingham, for kind help.

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