0273-2289/04/118/0089/$25.00

# Derepressed 2-Deoxyglucose-Resistant Mutants of *Aspergillus niger* With Altered Hexokinase and Acid Phosphatase Activity in Hyperproduction of β-Fructofuranosidase

**B. Ashokkumar, S. R. Senthilkumar, and P. Gunasekaran***

*Department of Microbial Technology, School of Biological Sciences, Centre for Excellence in Genomic Sciences, Madurai Kamaraj University, Madurai 625 021, India, E-mail: pguna@eth.net*

**Received April 25, 2003; Revised September 12, 2003; Accepted September 18, 2003**

## Abstract

*Aspergillus niger* NRRL330 produces extracellular β-fructofuranosidase (Ffase), and its production is subject to repression by hexoses in the medium. After ultraviolet mutagenization and selection, seven derepressed mutants resistant to 2-deoxyglucose (2-DG) were isolated on Czapek's minimal medium containing glycerol. One of the mutants, designated DGRA-1, produced higher levels of Ffase. A considerable difference occurred in the mutants with reference to hexokinase and intracellular acid phosphatase activities. The hexokinase activity of the mutant DGRA-1 (0.69 U/mg) was 1.8-fold higher than the wild type (0.38 U/mg). Intracellular acid phosphatase activity of the mutant DGRA-1 (0.83 U/g of mycelia) was twofold higher than that of the wild type (0.42 U/g of mycelia), suggesting that phosphorylation and dephosphorylation steps could attribute to the 2-DG resistance of *A. niger*. However, additional mutations could account for the increased production of Ffase in the mutant DGRA-1.

**Index Entries:** *Aspergillus niger*; hexokinase; acid phosphatase; β-fructofuranosidase; 2-deoxyglucose.

## Introduction

β-Fructofuranosidase (EC 3.2.1.26) (Ffase) is an invertase, which hydrolyzes the nonreducing disaccharide sucrose and produces equimolar amounts of glucose and fructose. Ffase is one of the most widely used enzymes in confectioneries, pharmaceuticals, and food industries especially

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

in the preparation of jams and candies, and as artificial sweeteners *(1)*. The production of invertase from a variety of filamentous fungi, such as *Aspergillus*, *Neurospora*, *Penicillium*, and *Aureobasidium*, has been reported, and it has been extensively studied in yeast and *Neurospora* sp. *(2)*.

The method of mutagenization by rational selection has been widely employed to obtain cultures with improved enzyme production. Improving the productivity of fungi in this way could be made considerably efficient by combining the selection of highly active mutants with their resistance to 2-deoxyglucose (2-DG) *(3)*. 2-DG is a glucose analog, which is readily phosphorylated by fungal hexokinases. The resulting compound, 2-deoxyglucose-6-phosphate, accumulates in the cytoplasm and inhibits the growth of yeasts and filamentous fungi by repressing early glycolytic enzymes *(4)*. Many mutant strains showing resistance to 2-DG in yeast and filamentous fungi have been isolated with a relatively large number of derepressed and hyperproductive mutants with a relatively shorter investment time. The productivity of enzymes such as pectinase in *Aspergillus niger (5)*, glucoamylase in *Rhizopus (6)*, and cellulase in *Trichoderma (7)* was improved by the selection of DG-resistant strains that were less sensitive to catabolite repression. In *Kluyveromyces lactis*, mutants devoid of hexokinase showed relief from catabolite repression of several enzymes *(8)*.

In our previous work, we used 2-DG as an antimetabolite in order to isolate 2-DG-resistant mutants of *A. niger* that overproduced Ffase *(3)*. One such mutant, DGRA-1, exhibited higher Ffase activity than the wild type. In the present study, we characterized the mutant DGRA-1 regarding 2-DG resistance and Ffase production in submerged fermentation (SmF) conditions.

## Materials and Methods

### Microorganism

*A. niger* NRRL330 was obtained from Northern Regional Research Laboratory (NRRL) (Peoria, IL), propagated on potato dextrose agar (PDA) medium at 35°C, and maintained at 4°C.

### Mutagenesis and Mutant Selection

Czapek's solution was used as a basal minimal medium (MM) containing 30 g/L of glycerol (MMG) as the carbon source and 10 g/L of 2-DG (MMGDG). Fresh conidia of *A. niger* NRRL330 in distilled water were subjected to ultraviolet (UV) (2 μJ/[$mm^2$·s]) irradiation from a distance of 15 cm, and the UV dose was adjusted to a survival rate of approx 10% for a suspension having a spore count of $10^7$ per Petri dish. Irradiated spore suspension (1 mL) was inoculated in 9 mL of MMGDG broth and incubated at 30°C in a rotary shaker (190 rpm) for 4 h. After incubation, 0.1 mL of the surviving spores was inoculated on MMGDG plates and incubated for 9 d. For selection of stable DG-resistant mutant strains, the spores were repeat-

edly subcultured on MMGDG plates, and selected colonies were picked up and maintained on PDA. Colonies showing good growth on MMGDG plates were selected and screened for Ffase production.

### *Fermentation Conditions*

The fermentation medium for submerged culture consisted of the following: 100 g/L of $(NH_4)_2SO_4$, 20 g/L of $KH_2PO_4$, 0.05 g/L of $FeSO_4$, 5 g/L of $MgSO_4 \cdot 7H_2O$, 100 g/L of sucrose, 10 g/L of urea, and 5 g/L of yeast extract with an initial pH of 5.0. Erlenmeyer flasks (250 mL) containing 50 mL of medium were inoculated with $1 \times 10^5$ spores/mL and incubated at 30°C in a rotary shaker (190 rpm) for 5 d. At 24-h intervals, samples (independent flasks) were removed, and the mycelia were separated by filtration through Whatman no. 1 filter paper. The filtrate was centrifuged at 8000*g* for 15 min, and the clear supernatant was assayed for Ffase activity.

### *Extraction of Intracellular Enzymes*

The fungal mycelia were washed three times with distilled water. The intracellular enzyme was recovered from the mycelia by grinding with an equal amount of acid-washed sand in 0.02*M* acetate buffer (pH 5.0) in a chilled mortar. The fungal debris and sand were removed by centrifuging at 5000*g* for 15 min, and the resulting supernatant was assayed for Ffase, hexokinase, and acid phosphatase activity.

### *Analytical Methods*

Ffase activity was determined by measuring the reducing sugars released by the hydrolysis of sucrose. Suitably diluted extract (0.1 mL) was mixed with 0.2 mL of 1*M* sucrose in 0.05*M* sodium acetate buffer (pH 5.0). The reaction was carried out at 37°C for 15 min and stopped by boiling for 10 min. The reducing sugars released in the reaction mixture were assayed by the Somogyi method *(9)*. One unit of Ffase activity was defined as the amount of enzyme required to release 1 µmol of reducing sugars equivalent/h under the assay conditions.

Hexokinase assay was based on the reduction of $NAD^+$ through a coupled reaction with glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and measured by the increase in absorbance at 340 nm *(10)*. For measuring hexokinase activity, 0.67*M* glucose in 0.05*M* Tris-HCl buffer (pH 8.0) with 13.3 m*M* $MgCl_2$, 16.5 m*M* adenosine triphosphate, 6.8 m*M* NAD, and 1.7 U of glucose-6-phosphate dehydrogenase was incubated at 30°C. The reaction was initiated by the addition of 0.1 mL of diluted hexokinase solution, and the increase in $A_{340}$ for 3 to 4 min was recorded. One unit of hexokinase activity reduces 1 µmol of $NAD^+$/min at 30°C and pH 8.0 under the specified conditions. Hexokinase activity was calculated using the following equation:

$$\text{U/mg protein} = (\Delta A_{340}/\text{min})/(6.22 \times \text{mg enzyme/mL reaction mixture})$$

Acid phosphatase activity was determined by measuring the quantity of *p*-nitrophenol released by the hydrolysis of *p*-nitrophenyl phosphate *(11)*. Culture supernatant (0.1 mL) was mixed with 0.2 mL of 10 m*M p*-nitrophenyl phosphate in citrate buffer (pH 4.0). The reaction was carried out at 37°C for 10 min and stopped by adding 1 mL of 1*M* NaOH. The concentration of liberated *p*-nitrophenol was determined at 405 nm. One unit of phosphatase activity was defined as the amount of enzyme releasing 1 µmol of either *p*-nitrophenol or inorganic phosphate/min.

The fungal mycelia in SmF were filtered and dried overnight at 80°C, and the biomass dry weight was determined. Protein concentration was determined by the Lowry method *(12)* with bovine serum albumin as the standard.

## Results and Discussion

The growth and production of Ffase in basal media supplemented with carbohydrates (1%) such as sucrose and glucose was studied (Fig. 1). In basal medium (control), the level of Ffase was 7.8 U/(mL·h) in 120 h of fermentation, and this is regarded as the constitutive level of Ffase produced by *A. niger* in SmF. A considerable increase in Ffase activity was observed with the culture grown for 120 h in basal medium supplemented with sucrose (54 U/[mL·h]). However, the glucose-grown culture did not show any significant increase in the level of Ffase (10.2 U/[mL·h]). When the glucose concentration was increased to 3% (w/v), the level of Ffase decreased to 4.8 U/(mL·h) at 120 h. These results show that the addition of glucose to the basal medium repressed the productivity of Ffase by *A. niger*. Similarly, the glucoamylase activity of *Aspergillus oryzae* was decreased when the glucose concentration was increased to 2% *(13)*, and pectinase from *A. niger* was repressed by glucose (5%) in SmF *(14)*. The production of other hydrolytic enzymes such as cellulase *(15)*, polygalacturonase, and pectin esterase was also repressed in SmF processes by glucose in the medium *(16)*.

Nonmetabolizable repressors such as 2-DG have been used to isolate selectively a relatively large number of mutants derepressed and hyperproductive with respect to repressible enzymes *(17)*. Many mutant strains showing DG resistance in yeast and filamentous fungi have been isolated, and possible DG-resistant mechanisms have been studied. They are as follows: a new phosphatase specific for DG-6-phosphate induced and hydrolyzed to prevent intracellular accumulation of the inhibitory DG-6-phosphate *(18)*, induction of a defective hexokinase to avoid the phosphorylation of 2-DG *(19)*, and an altered transport system for glucose *(20)*.

The antimetabolite 2-DG acts simultaneously as catabolite repressor of repressible enzymes and as an inhibitor of fungal growth *(7)*. In our experiment, 2-DG was used at a concentration of 10 g/L with glycerol (3%), which effectively repressed but did not completely inhibit the growth of fungi. Therefore, to obtain derepressed mutants, the conidia of *A. niger*

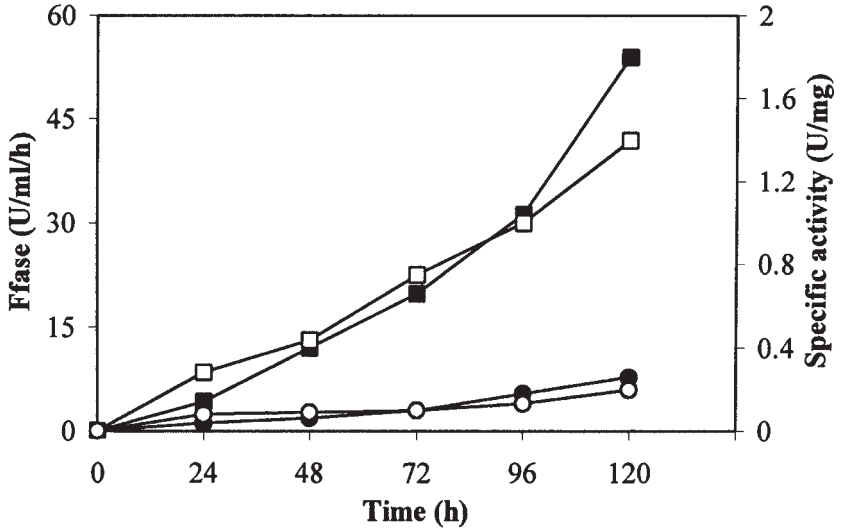


Fig. 1. Kinetics of Ffase production by *A. niger* (wild type) in SmF with sucrose and glucose (1%): (■) Ffase activity in sucrose; (●) Ffase activity in glucose; (□) specific activity in sucrose; (○) specific activity in glucose.

Table 1
Production of Ffase by DG-Resistant Mutants of *A. niger* in SmF

| Strain | Ffase productivity (U/[mL·h]) | Biomass (g/L) | Specific activity (U/mg) |
|---|---|---|---|
| DGRA-1 | 798 | 36.0 | 4.2 |
| DGRA-3 | 198 | 42.2 | 1.4 |
| DGRA-5 | 300 | 43.0 | 2.3 |
| DGRA-7 | 198 | 48.4 | 1.4 |
| DGRA-9 | 102 | 42.8 | 0.8 |
| DGRA-12 | 78 | 40.8 | 0.6 |
| DGRA-14 | 408 | 48.0 | 2.4 |
| Parent | 408 | 36.2 | 3.2 |

were exposed to UV irradiation, and the mutant strains showing good growth on MMGDG (10 g/L) plates were selected and termed DG-resistant mutants DGRA-1, DGRA-2, and so on. A total of 14 mutant strains were obtained by the first selection. Among them, seven showed poor growth on MMGDG plates, and the remaining seven stably maintained DG resistance after several subcultures on PDA. They were then screened for Ffase productivity in SmF conditions. Of seven mutants, DGRA-1 and DGRA-14 showed enhanced Ffase productivity, whereas the remaining five showed decreased productivity, compared to the parent strain. As shown in Table 1, the mutant DGRA-1 exhibited 2.3-fold higher Ffase productivity (798 U/[mL·h]) than the parent strain (348 U/[mL·h]). The increased

Table 2
Production of Hexokinase Activity
by Wild-Type and DG-Resistant Mutants
of *A. niger* in SmF

| | Intracellular hexokinase activity | |
|---|---|---|
| Strain | U/mg of protein | % |
| Wild type | 0.38 | 100 |
| DGRA-1 | 0.69 | 182 |
| DGRA-3 | 0.32 | 84 |
| DGRA-5 | 0.46 | 121 |
| DGRA-7 | 0.36 | 95 |
| DGRA-9 | 0.28 | 74 |
| DGRA-12 | 0.19 | 50 |
| DGRA-14 | 0.64 | 168 |

yield of Ffase production by DGRA-1 was constant during subsequent subcultures.

### *Hexokinase Activity of Wild-Type and DGR Mutant of* A. niger

Induced hexokinase activities in mutants to avoid the phosphorylation of 2-DG have been shown to be a possible mechanism of DG resistance *(19)*. Rose et al. *(21)* showed that triggering glucose repression is directly associated with the activity of hexokinases. Mutants devoid of hexokinase showed relief from carbon catabolite repression in *K. lactis (8)*. In our study, considerable divergencies occurred in the level of hexokinase activity produced by 2-DG-resistant mutants of *A. niger*. Among the seven mutants, it was less in DGRA-3, DGRA-9, and DGRA-12 in comparison with the wild-type strain, whereas DGRA-1, DGRA-5, DGRA-7, and DGRA-14 showed increased hexokinase activity. As shown in Table 2, the mutants DGRA-1 and DGRA-14 exhibited greater than 1.8- and 1.7-fold higher hexokinase activity, respectively (0.69 and 0.64 U/mg of protein), than the parent strain (0.38 U/mg of protein). Thus, increased levels of hexokinase activity of mutants seem to be compatible with the possible mechanism of induced levels of hexokinase to exert resistance against glucose repression in *A. niger*.

### *Acid Phosphatase Activity of Wild-Type and DGR Mutant of* A. niger

Increased activity of intracellular acid phosphatase is reported to be a mechanism of resistance to DG. A new phosphatase specific for DG-6-phosphate is induced and dephosphorylates to prevent intracellular accumulation of the inhibitory DG-6-phosphate *(18)*. The levels of intracellular acid phosphatase activity produced by a wild-type and DGR mutant of *A. niger* in SmF is shown in Table 3. Moreover, the production of acid phosphatase by the DG-resistant mutant DGRA-1 of *A. niger* was twofold higher (0.83 U/g of mycelia) compared with the wild type (0.42 U/g of

Table 3
Production of Acid Phosphatase
by Wild-Type and DG-Resistant Mutants of *A. niger* in SmF

| | Acid phosphatase activity | | | |
|---|---|---|---|---|
| | Intracellular | | Extracellular | |
| Strain | U/g | % | U/mL | % |
| Wild type | 0.42 | 100 | 0.03 | 100 |
| DGRA-1 | 0.83 | 198 | 0.06 | 200 |
| DGRA-3 | 0.28 | 67 | 0.02 | 67 |
| DGRA-5 | 0.50 | 119 | 0.025 | 83 |
| DGRA-7 | 0.40 | 95 | 0.02 | 67 |
| DGRA-9 | 0.22 | 52 | 0.018 | 60 |
| DGRA-12 | 0.11 | 26 | 0.013 | 43 |
| DGRA-14 | 0.55 | 131 | 0.045 | 150 |

mycelia). Similarly, Heredia and Sols *(22)* obtained a *Saccharomyces cerevisiae* mutant with 10 times higher intracellular phosphatase activity. Therefore, a high activity of acid phosphatase produced by the DGR mutant of *A. niger* may be involved in the dephosphorylation of toxic DG-6-phosphate to prevent its intracellular accumulation.

## Conclusion

*A. niger* was mutagenized to obtain strains resistant to 2-DG. The mutant DGRA-1 exhibited greater than 2.3-fold higher Ffase productivity than its parent. Hexokinase and intracellular acid phosphatase activity of the mutant DGRA-1 was greater than 1.8- and 2-fold higher, respectively, when compared with the parent. Therefore, it is possible that a high activity of hexokinase and acid phosphatase produced by the DGR mutant of *A. niger* (DGRA-1) may be involved in derepression by altered phosphorylation of glucose or by dephosphorylation of toxic DG-6-phosphate to prevent its intracellular accumulation. The mutant DGRA-1 possibly carries multiple mutations leading to increased production of Ffase.

## Acknowledgment

We gratefully acknowledge the UGC (P. no. 3-25/98 [SR-II]), New Delhi, for financial support.

## References

1. Klein, R. D., Deibel, M. R., Sarcich, J. L., Zucher-Neely, H. A., Reardon, J. M., and Heinrikson, R. L. (1989), *Prep. Biochem*. **19,** 283–319.
2 Chen, J. S., Saxton, J., Hemming, F. W., and Peberdy, J. F. (1996), *Biochim. Biophys. Acta* **1296,** 207–218.
3. Ashokkumar, B. and Gunasekaran, P. (2002), *Indian J. Exp. Biol*. **40,** 1032–1037.

4. Moore, D. (1981), *N. Phytol*. **87,** 487–515.
5 Antier, P., Minjares, A., Roussos, S., and Viniegra-Gonzalez, G. (1993), *Biotechnol. Adv*. **11,** 429–440.
6 Tani, Y., Fuji, A., and Nishise, H. (1988), *J. Ferment. Bioeng*. **66,** 545–551.
7 Labudova, I. and Farkas, V. (1983), *FEMS Microbiol. Lett*. **20,** 211–215.
8. Goffrini, P., Ficarelli, A., and Ferrero, I. (1995), *Microbiology* **141,** 441–447.
9. Somogyi, M. J. (1951), *J. Biol. Chem.* **195,** 19–23.
10. Abrahao-Neto, J., Infanti, P., and Vitolo, M. (1996), *Appl. Biochem. Biotechnol*. **57/58,** 407–412.
11 Golovan, S., Wang, G., Zhang, J., and Forsberg, C. W. (2000), *Can. J. Microbiol*. **46,** 59–71.
12 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265–275.
13. Facciotti, M. C. R., Kilikian, B. V., Schmidell, W., and Fachini, E. R. (1989), *Rev. Microbiol.* **20,** 108–114.
14 Aguilar, G. and Huitron, C. (1987), *Enzyme Microb. Technol.* **9,** 690–696.
15 Gokhale, D. V., Patil, S. G., and Bastawade, K. B. (1992), *Appl. Biochem. Biotechnol*. **37,** 11–17.
16. Maldonado, M. C., Strasser De Saad, A. M., and Callieri, D. (1989), *Curr. Microbiol*. **18,** 303–306.
17 Fiedurek, J., Paszczynski, A., Ginalska, G., and Ilczuk, Z. (1987), *Zentralbl. Mikrobiol*. **142,** 407–412.
18 Martin, M. and Heredia, C. F. (1977), *FEBS Lett*. **83,** 245–248.
19 Lobo, Z. and Maitra, P. K. (1977), *Mol. Gen. Genet*. **157,** 297–300.
20 Moore, D. and Devadatham, M. S. (1979), *Biochim. Biophys. Acta* **550,** 515–526.
21 Rose, M., Albig, W., and Entian, K. D. (1991), *Eur. J. Biochem*. **199,** 511–518.
22. Heredia, C. F. and Sols, A. (1964), *Biochim. Biophys. Acta* **86,** 224–228.