

β-fructofuranosidase production by 2-deoxyglucose resistant mutants of
Aspergillus niger in submerged and solid-state fermentation

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Aspergillus niger produces extracellular β-fructofuranosidase under submerged (SmF) and solid state fermentation (SSF) conditions. After UV mutagenesis of conidiospores of A. niger, 2-deoxyglucose (10 g/l) resistant mutants were isolated on Czapek’s minimal medium containing glycerol as a carbon source and the mutants were examined for improved production of β-fructofuranosidase in SmF and SSF conditions. One of such mutant DGRA-1 overproduced β-fructofuranosidase in both SmF and SSF conditions. In SmF, the mutant DGRA-1 showed higher β-fructofuranosidase productivity (110.8 U/hr) than the wild type (48.3 U/hr). While in SSF the same strain produced 332 U/hr of β-fructofuranosidase, 2 times higher than that of wild type (154.2 U/hr). In SmF, both wild type and mutants produced relatively low level of β-fructofuranosidase in medium containing sucrose with glucose than from the sucrose medium. However in SSF, the DGRA-1 mutant grown in sucrose and sucrose + glucose did not show any difference with respect to β-fructofuranosidase production. These results indicate that the catabolite repression of β-fructofuranosidase synthesis is observed in SmF whereas in SSF such regulation was not prominent.

β-fructofuranosidase (EC.3.2.1.26) catalyses the hydrolysis of sucrose into glucose and fructose by recognizing the fructose moiety of sucrose. It is extensively used in confectioneries, food industries as artificial sweeteners and in pharmaceuticals. Solid-state fermentation (SSF) has potential advantages over the submerged state fermentation (SmF) with respect to simplicity in operation, high productivity fermentation, less favorable for growth of contaminants and concentrated product formation. Certain investigations revealed that the strains used in SSF are less efficient in SmF. Several mutant strains of A. niger for pectinase production have been isolated, that showed improved efficiency in SSF but not in SmF and vice versa. However, the composition of the medium used in SmF and SSF were same. β-fructofuranosidase production under SmF and SSF conditions was improved through media optimization approaches. From the studies of fungal carbon metabolism, much useful information has come during the isolation of toxic sugar analog resistant mutants. One such analog is 2-deoxyglucose (DG), 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. A variety of 2-DG resistant mutants have been found in S. cerevisiae with reduced phosphorylation of 2-DG and induction of a new phosphatase specific for DG-6-phosphate is induced and dephosphorylates to prevent the intracellular accumulation of the inhibitory DG-6-phosphate. Many 2-DG resistant mutants appear to have pleiotropic regulatory alterations in the activity of enzymes such as invertase, α-glucosidases and malate dehydrogenases.

In filamentous fungi, 2-DG resistant mutants of Neurospora crassa were isolated and the productivity of pectinase in A. niger, glucoamylase in A. terreus and cellulase in Trichoderma reesei were improved by the selection of DG-resistant strains. In this paper, we report the isolation of DG-resistant mutants of A. niger using UV mutagenesis followed by the selection of 2-deoxyglucose resistant colonies for overproduction of β-fructofuranosidase in SmF and SSF.

Materials and Methods

Microorganism and culture conditions
Aspergillus niger 330 was obtained from Northern Regional Research Laboratory, Peoria, Illinois, USA.
propagated on potato dextrose agar medium (PDA) at 35°C and stored at 4°C.

Mutagenesis and mutant selection

Czapek's solution as basal minimal medium (MM) containing 30 g/l of glycerol (MMG) as the carbon source with 10 g/l of 2-deoxyglucose (MMGDG) was used. Fresh conidia of A. niger 330 in distilled water was subjected to UV (2 μm m^-2 s^-1) irradiation from a distance of 15 cm and the UV dose was adjusted to a survival rate approximately of 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 rotatory shaker (190 rpm) for 4 hr. After incubation, 0.1 ml of surviving spores were inoculated on MMGDG agar plates and incubated for 9 days. For selection of stable DG-resistant mutant strains, the spores were repeatedly subcultured on MMGDG plates again, and selected colonies were picked up and maintained on PDA. Colonies showing good growth on MMGDG plates were selected and examined for β-fructofuranosidase production.

Fermentation conditions

The optimized fermentation medium for submerged culture consisted (g/l) of: (NH₄)₂SO₄, 100; KH₂PO₄, 20; FeSO₄, 0.05; MgSO₄·7H₂O, 5; sucrose, 100; urea, 10 and yeast extract, 5, initial pH 5. Erlenmeyer flasks (250 ml) containing 50 ml of medium were inoculated with 1 x 10⁷ spores/ml and incubated at 30°C in a rotatory shaker (190 rpm) for 5 days. At every 24 hr intervals, samples (independent flasks) were removed and the mycelia were separated by filtration through Whatman No.1 filter paper. The filtrate was centrifuged at 10,000 rpm for 15 min and the clear supernatant was assayed for β-fructofuranosidase activity.

For solid state fermentation, the optimized medium consisted (g/l) of: (NH₄)₂SO₄, 10; KH₂PO₄, 40; FeSO₄, 0.05; MgSO₄·7H₂O, 2; sucrose-200; urea, 10 and yeast extract, 10. The media components were dissolved in 80% of the total volume and mixed with the sugarcane bagasse (10g) in Erlenmeyer flasks and inoculated at a concentration of 1 x 10⁵ spores/g of support material. The flasks were incubated statically at 30°C. At 24 hr intervals, individual flasks were removed and the contents were extracted with 15 ml of 0.05 M sodium acetate buffer by mechanical squeezing and the extract was centrifuged at 10,000 rpm for 15 min. The clear supernatant was assayed of β-fructofuranosidase activity.

Analytical methods

β-fructofuranosidase activity was determined by measuring the reducing sugars released by the hydrolysis of sucrose. For assay, 0.1 ml of suitably diluted extract was mixed with 0.2 ml of 1M sucrose in 0.05M sodium acetate buffer (pH 5). 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 Somogyi method. One unit of β-fructofuranosidase activity was defined as the amount of enzyme required to release one μmole of reducing sugars equivalent per minute under the assay conditions.

Protease activity was determined as described by Longo et al. For assay, a reaction mixture containing 0.3 ml of 0.5% Azocasein in sodium acetate buffer (pH 5), 0.1 ml of 10 mM CaCl₂, 0.2 ml of enzyme solution and 0.4 ml of sodium acetate buffer (pH 5) was incubated at 37°C for 30 min. The reaction was then stopped with 20% trichloroacetic acid. The mixture was centrifuged at 10,000 rpm for 5 min and the filtrate was neutralized with 1.8 N NaOH and absorbance read at 425 nm. One unit of protease activity was defined as the amount of enzyme produces an increase in absorbance to 0.5 under the assay conditions.

The fungal mycelia in SmF was filtered and dried for overnight at 80°C and the biomass dry weight was determined. In SSF, due to the interference of bagasse in protein measurements by Lowry or Kjeldhal technique, biomass could not be estimated accurately. Soluble proteins in the culture filtrates were estimated by the method of Lowry et al., with bovine serum albumin as a standard.

Results

Isolation of 2-deoxyglucose resistant mutants of A. niger

To determine the minimum inhibitory concentration (MIC) of 2-DG for A. niger 330, it was cultivated on MMG containing different concentrations of 2-DG (1 g/l -50 g/l). A. niger 330 grew well on MMG plates, but the addition of 2-DG (10 g/l) to MMG plates resulted in marked inhibition of the growth of this strain. The diameter of mycelial growth of the A. niger colony was 30 mm on MMG plate while it was 8 mm on MMGDG plate (10 g/l). The mutants were selected after UV-irradiation of wild type strain and then plating in the medium MMGDG (10 g/l). Some mutant strains showed good growth on MMGDG.
(10 g/l) plates were selected and they were termed as DG resistant mutants DGRA-1, DGRA-2 and so on. A total of 14 mutants were obtained by the first selection and were screened further. Among the 14 mutants, 7 strains showed poor growth on MMGDG plates. Other 7 strains showed luxurious growth on MMGDG plates and stably maintained DG-resistance after several subcultures on PDA. Therefore, these 7 strains were examined for β-fructofuranosidase productivity in SmF and SSF conditions.

β-fructofuranosidase production by DGRA mutant strains in SmF

The efficiency of mutants was compared with parent strain for β-fructofuranosidase production in SmF conditions. When compared to the parent strain, among the 7 mutants DGRA-1 showed enhanced β-fructofuranosidase productivity and 5 other strains showed decreased productivity. As shown in Table 1, the mutant DGRA-1 exhibited over 2-fold higher β-fructofuranosidase productivity (110.8 U/l/hr) than the parent strain (48.3 U/l/hr). Despite resistance to 2-DG, the mutant DGRA-14 produced equal amount of β-fructofuranosidase to that of parent strain. The increased productivity of β-fructofuranosidase by the mutants would be attributed to a) mutation leading to the increased rate of synthesis of β-fructofuranosidase or b) mutation leading to the reduced level or loss of protease production resulting in higher stability of the β-fructofuranosidase. To confirm the above possibilities the protease level in the culture filtrate was estimated. Under SmF fermentation condition, A. niger wild type and the mutant DGRA-1 did not produce detectable amount of protease activity in the culture filtrate (data not shown). Therefore, the enhanced production of β-fructofuranosidase by the mutant cannot be correlated with protease activity.

β-fructofuranosidase production by the DGRA mutant strains in SSF

The efficiency of DG resistant mutants of A. niger were examined for β-fructofuranosidase productivity in SSF and shown in Table 2. Compared to the parental strain, the mutants DGRA-1, DGRA-5, DGRA-14 (322, 200, 310 U/l/hr respectively) showed increased productivity of β-fructofuranosidase and some of them namely DGRA-7, DGRA-9 (159 and 173 U/l/hr) had similar productivity, and DGRA-3, DGRA-12 had decreased productivity (141 and 89 U/l/hr). The mutants DGRA-1 and DGRA-14 exhibited over 2-fold higher β-fructofuranosidase productivity (322 and 310 U/l/hr) than the parental strain (154.2 U/l/hr). The mutants DGRA-5, DGRA-7 and DGRA-9 had slightly higher β-fructofuranosidase production (200, 159 U/l/hr and 173 U/l/hr respectively) than their parent strain. During the analysis of protease activity in the culture filtrate of wild type (5.2 U/ml) and DG

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resistant mutants (2.8 to 6.1 U/ml), protease was detected but their levels were not significant. Therefore the reason for enhanced level of β-fructofuranosidase by the mutants could not be attributed to the improved rate of synthesis of the enzyme.

Comparison of DGRA mutants for β-fructofuranosidase production in SmF and SSF

The relative β-fructofuranosidase productivity of the DGRA mutants over their parent strain in SmF and SSF was compared. The mutants DGRA-1 and DGRA-12 showed increased and decreased productivity of β-fructofuranosidase respectively in both SmF and SSF. The kinetics of β-fructofuranosidase production by the mutant DGRA-1 in SmF and SSF was studied and compared with its parent strain (Fig. 1). The mutant DGRA-1 produced a maximum of 110.8 U/I/hr at 120 hr while the parent produced only 48.3 U/I/hr in SmF. Similarly the mutant DGRA-1 produced 322 U/I/hr at 72 hr in SSF while its parent produced only 154.2 U/I/hr. Thus the mutant DGRA-1 showed an improved productivity of β-fructofuranosidase in both SmF and SSF. In SSF, the mutants DGRA-5 and DGRA-14 showed increased productivity while DGRA-3 and DGRA-7 and DGRA-9 produced equal amount of β-fructofuranosidase compared to their parent strain. None of the strain other than DGRA-1 showed increased productivity in SmF. On the contrary the strains DGRA-3, DGRA-7 and DGRA-9 showed decreased productivity than their parent strain.

It is interesting to note that the strains DGRA-5 and DGRA-14, which did not show much difference in β-fructofuranosidase productivity with their parent strain in SmF, while increased productivity was shown in SSF. Similarly, the β-fructofuranosidase productivity of the mutants DGRA-3, DGRA-7 and DGRA-9 were comparable to their parent strain in SSF, but decreased in SmF. Therefore these results suggested that mutations that would influence the enzyme production in SSF and SmF could be different. Possibly the mutant DGRA-1 and DGRA-12 might carry multiple mutations leading to increased/decreased production of β-fructofuranosidase in both SSF and SmF.

β-fructofuranosidase synthesis in A. niger is inducible β-fructofuranosidase production by A. niger in SmF with medium containing glucose and sucrose was studied. Glucose grown culture of A. niger 330 and DGRA-1 mutant produced low level (5 to 7 U/I/hr) of β-fructofuranosidase in SmF while in sucrose medium the strains produced high level of β-fructofuranosidase (16.7 to 17.0 U/I/hr) at 24 hr (Fig. 2). Compared to the culture in SmF, these strains produced a higher level of β-fructofuranosidase with glucose (33 to 35 U/I/hr) and sucrose (105 to 119 U/I/hr) in SSF at 24 hr. Therefore the β-fructofuranosidase production in A. niger seems to be inducible by the substrate sucrose. Earlier studies clearly indicated that the β-fructofuranosidase production by A. nidulans and A. niger was inducible by sucrose and raffinose.

Carbon catabolite repression of β-fructofuranosidase synthesis

In order to find out the inducible β-fructofuranosidase synthesis is regulated by catabolite repression in SmF, the culture was grown in medium containing

![Fig. 1](image1)

**Fig. 1** — Kinetics of β-fructofuranosidase production by A. niger (wild type) and 2-DG resistant mutant (DGRA-1) in SmF and SSF. [Wild type (●) and mutant (▲); in SmF; Wild type (▲) and mutant (■) in SSF]

![Fig. 2](image2)

**Fig. 2** — Effect of sucrose and glucose on β-fructofuranosidase synthesis by A. niger (wild type) and DG resistant mutant (DGRA-1) in SmF (A) and SSF (B). [Wild type grown on medium with 3% glucose (●) and sucrose (▲); DGRA-1 grown on medium with 3% glucose (●) and sucrose (■)]
enzyms are produced using both SSF and SmF with fungal strains. SSF is generally considered more suitable for rendering higher yields of hydrolases. It has also been reported that the mutant strains of A. niger for pectinase production showed improved efficiency in SSF but not in SmF and vice versa. Therefore, the mutants were grown in solid culture to study their efficiency in SSF and to compare the productivity of mutants in SmF. Many mutant strains showing DG-resistance in yeast and filamentous fungi have been isolated and possible DG-resistant mechanisms were studied.

In the present study, a series of 7 mutants of A. niger which are resistant to 2-deoxyglucose were obtained using UV mutagenesis and screened for \( \beta \)-fructofuranosidase production in SmF and SSF. Among the 7 mutants, DGRA-1 exhibited twofold higher \( \beta \)-fructofuranosidase production under both SmF (110.8 U/l/hr) and SSF (322 U/l/hr) conditions than the parental strain (48.3 U/l/hr in SmF and 154.2 U/l/hr in SSF). One more mutant, DGRA-14 also showed enhanced productivity of \( \beta \)-fructofuranosidase in SSF (310 U/l/hr). The improved production of hydrolytic enzymes can be achieved by the induction of protease-negative mutants leading to indirectly increased productivity of enzymes by reducing its degradation. In this study, the analysis of protease activity in the culture filtrate of SmF by wild type and mutant DGRA-1 did not produce detectable amount of protease activity, while in SSF protease was detected but their levels were not significant. In SSF, protease activity was in the culture filtrate of DG resistant mutants was in the range of 2.8 to 6.1 U/ml, but for wild type it was 5.2 U/ml. Therefore the enhanced productivity of \( \beta \)-fructofuranosidase by the mutants cannot be correlated with protease activity.

Glucose grown culture of A. niger wild type and DGRA-1 mutant produced low level of \( \beta \)-fructofuranosidase while the production of \( \beta \)-fructofuranosidase was comparatively higher in sucrose medium. Therefore, the \( \beta \)-fructofuranosidase production in A. niger seems to be inducible by the substrate sucrose. However in SSF comparatively higher level of \( \beta \)-fructofuranosidase was produced in the medium with glucose. This could be attributed to the presence of inducible substances (residual sucrose) present or released in the support material bagasse. Additionally, both the cultures produced relatively low level of \( \beta \)-fructofuranosidase in medium supplemented with sucrose and glucose than from the sucrose medium. However in SSF, the DGRA-1 mutant grown in

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**Discussion**

Solid-state fermentation (SSF) holds tremendous potential for the production of enzymes than submerged state fermentation (SmF) with respect to simplicity in operation, high productivity fermentation, less favorable for the growth of contaminants and concentrated product formation etc. Several authors have suggested that SSF productivity for various types of hydrolytic enzymes are higher than the one obtained by SmF. Industrially, several hydrolytic

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**Fig. 3**—Effect of sucrose and sucrose+glucose on \( \beta \)-fructofuranosidase synthesis by A. niger (wild type) and DG resistant mutant (DGRA-1) in SmF (A) and SSF (B). (Wild type grown on medium with sucrose—\( \bullet \) and sucrose+3% glucose—\( \bigcirc \) DGRA-1 grown on medium with sucrose—\( \bullet \) and sucrose+3% glucose—\( \bigcirc \)).

**A**

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**B**

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Sucrose and sucrose+3% glucose. The culture produced relatively low level of \( \beta \)-fructofuranosidase (19 to 22 U/l/hr) at 120 h in the medium supplemented with sucrose and glucose than from the sucrose medium (48.3 to 97.5 U/l/hr). However in SSF the culture grown in sucrose (154.2 to 282.5 U/l/hr) and sucrose+glucose (128 to 274.5 U/l/hr) did not show any difference with respect to \( \beta \)-fructofuranosidase production (Fig 3). These results suggested that the catabolite repression of \( \beta \)-fructofuranosidase synthesis was observed in SmF condition whereas in SSF such regulation was not prominent. Earlier, such a difference between SmF and SSF was also reported with respect to pectinase production by A. niger.
sucrose and sucrose + glucose did not show any difference with respect to β-fructofuranosidase production. The similar behavior is already reported in Bacillus licheniformis in which the effect of catabolite repression was significantly minimized by SSF in the production of α-amylase \(^1\) and in pectinase production by A. niger \(^3\). This has been suggested as an advantage of SSF over SmF. Increased uptake of substrate coupled with rapid growth of the organism leading to the drop in the level of substrate concentration in the vicinity of cells are contributing factors for this phenomenon in SSF. From the results it can be concluded that the production of β-fructofuranosidase by A. niger \(^3\) and 2-DG resistant mutant DGRA-1 in SmF is subjected to carbon catabolite repression whereas such regulation was not prominent in SSF.

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References