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Mutagenesis and Analysis of Mold *Aspergillus niger* for Extracellular Glucose Oxidase Production Using Sugarcane Molasses*

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Abstract

Aspergillus niger ORS-4.410, a mutant of *A. niger* ORS-4, was generated by repeated ultraviolet (UV) irradiation. Analysis of the UV treatment dose on wild-type (WT) *A. niger* ORS-4, conidial survival, and frequency of mutation showed that the maximum frequency of positive mutants (25.5%) was obtained with a 57% conidial survival rate after the second stage of UV irradiation. The level of glucose oxidase (GOX) production from mutant *A. niger* ORS-4.410 thus obtained was 149% higher than that for WT strain *A. niger* ORS-4 under liquid culture conditions using hexacyanoferrate (HCF)-treated sugarcane molasses (TM) as a cheaper carbohydrate source. When subcultured monthly for 24 mo, the mutant strain had consistent levels of GOX production (2.62 ± 0.51 U/mL). Mutant *A. niger* ORS-4.410 was markedly different from the parent strain morphologically and was found to grow abundantly on sugarcane molasses. The mutant strain showed 3.43-fold increases in GOX levels (2.62 ± 0.51 U/mL) using HCF-TM compared with the crude form of cane molasses (0.762 ± 0.158 U/mL).

Index Entries: Glucose oxidase; sugarcane molasses; submerged fermentation; *Aspergillus niger*; mutation.

Introduction

The flavoprotein glucose oxidase (GOX) (β-D-glucose oxygen 1-oxidoreductase, EC 1.1.3.4) from *Aspergillus niger* catalyzes the oxidation of

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β-D-glucose to gluconic acid, utilizing molecular oxygen as an electron acceptor with the simultaneous production of H_2O_2. GOX has several industrial applications, including quantitative determination of glucose in solution and body fluids such as blood and urine, generation of H_2O_2 for food preservation, and production of organic acids such as gluconic acid and its derivatives *(1,2)*. Several potential microorganisms, such as *A. niger*, *Penicillium* sp., *Talaromyces flavus*, and yeast, were screened for the possibility of commercial production of GOX *(3–7)*. The enzyme has mainly been observed as intracellular in *A. niger (8)*, whereas it is generally detected as extracellular from *Penicillium* sp. *(9)*. The enhanced production of extracellular GOX was attempted using a recombinant strain of *Saccharomyces cerevisiae (10,11)*. To economize the GOX fermentation process, extracellular GOX production in a fermentation medium is of prime importance. However, some of these applications prove uneconomical, owing to the higher cost during commercial preparation of the GOX.

A number of attempts have been made to select and improve strains for GOX production *(9,11,12)*, and a variety of carbohydrates, such as refined glucose, xylane, and corn steep liquor, have been proposed for production *(11,13,14)*. However, analysis of the mutagenesis and stability of the selected mutants must be elaborated in order to make the process viable in an industrial context. In addition, to economize the overall GOX fermentation process, the fermentation medium needs to be supplemented with cheaper ingredients, particularly the carbon substrate. Sugarcane industry byproducts such as molasses, which contain high concentrations of sugar and are readily available, have limited applications; that is, they are subjected to fermentation for the production of alcohol or used for animal feed.

The goal of the present study was to isolate a potent microbial strain from soil samples collected from sugarcane industry waste sites. The selection of a potential microbial strain was particularly based on its ability to produce extracellular GOX as well as to have effective growth of the selected strain over the crude and rectified forms of sugarcane molasses. The selected strain was further modified by mutagenesis using ultraviolet (UV) irradiation, and the stability of the selected mutant was monitored to analyze the consistency of the improved levels of GOX production. In addition, we attempted to evaluate the production levels of GOX using crude and clarified forms of sugarcane molasses as unconventional carbohydrate sources in the fermentation medium.

Materials and Methods

Isolation of Microorganism From Natural Site

A soil sample from sugarcane industry waste was collected, mixed with two parts of ddH_2O, and centrifuged at 850*g* at room temperature for 20 min. The clear supernatant was used for inoculating the yeast extract agar medium, which contained 10 g/L of glucose, 10 g/L of yeast extract,

15 g/L of agar, 1 g/L of NaCl, and 0.01 g/L of $MgSO_4 \cdot 7H_2O$. Growth of the microorganism was recorded after 72 h of incubation at 30°C. Seven distinct colonies appeared on the yeast extract agar medium, which were further analyzed for a semiquantitative test for GOX activity as described previously *(1)*. Briefly, the spores collected from the colonies were diluted and plated on medium containing 6 g/L of $NaNO_3$; 1.5 g/L of KH_2PO_4; 0.5 g/L of KCl; 0.5 g/L of $MgSO_4 \cdot 7H_2O$ with traces of $MnCl_2$, $ZnSO_4$, $CuSO_4$, and $FeSO_4$; 15 g/L of agar; 20 g/L of glucose as the carbon source; and 2.5 m*M* *o*-anisidine. After 40 h of incubation at 30°C, a solution containing 20 m*M* sodium phosphate buffer, pH 7.0; 0.1 *M* glucose; and 20 µg/mL of horseradish peroxidase (HRP) was added over the colonial growth. The GOX-containing colonies that turned brownish red were picked up for further analysis for GOX production. A colony that had a wider brownish red zone was picked and denoted as ORS-4. This selected colony was identified by the Indian Agricultural Research Institute as *A. niger* (ITCC 5231) and maintained on potato dextrose agar (PDA) slants at 4°C by periodic transfers.

UV Mutation and Selection of Mutants

The organism *A. niger* was grown on PDA plates for 72 h at 30°C before mutagenesis. The 3-d-old conidiospores of *A. niger* ORS-4 were suspended in 10 mL of sterile 50 m*M* phosphate buffer (pH 6.8) to a concentration of 1×10^9 conidiospores/mL in a sterile Petri dish. Suspended conidiospores were subjected to the two-step physical mutagenesis by UV irradiation (2.5 J/[m^2·s], distance of 0.69 m) at different times ranging from 1 to 12 min. After each treatment, the conidial survival was determined on PDA plates at 30°C, and the selection of mutants was performed on *o*-dianisidine containing double-layered agar plates as described by Petruccioli et al. *(9)*. Briefly, in the two-layered solid medium, the lower layer contained 10 g/L of glucose, 0.1 g/L of *o*-dianiside, 0.05 g/L (200 U/mg) of HRP, and 20 g/L of agar; the upper layer contained the basal medium in which $CaCO_3$ along with agar (20 g/L) was added to a concentration of 50 g/L instead of 40 g/L. To prevent heat inactivation, peroxidase was added to the agar at a temperature less than 50°C. After solidification, the plates were inoculated with serially diluted spores of *A. niger* to ensure 15–20 colonies per plate. Plates were then incubated at 30°C for 48 h. GOX activity was measured as the diameter of the brown halo of oxidized *o*-dianisidine around the colonies. The mutants, which showed the widest zone of diffusion as compared to the WT, were isolated and further characterized in the liquid culture medium.

Clarification of Sugarcane Molasses

Crude form cane molasses (CM) was found to contain high concentrations of heavy metals and other compounds that inhibited GOX fermentation; hence, it was treated with hexacyanoferrate (HCF) prior to use as described previously *(15)*. Briefly, the CM (1 kg, obtained from a local sug-

arcane mill) was diluted four to five times with deionized water and passed through a bed of activated charcoal for decolorization. HCF (3.8 m*M*) was added to the decolorized molasses at pH 4.0–4.5, followed by heating at 70–90°C for 15 min. The precipitate formed containing metallic complex was removed by filtration, and the filtrate was referred to as treated cane molasses (TM). The pH of clarified molasses was adjusted to 4.5 before its use for GOX fermentation.

Growth in Liquid Medium and Culture Conditions

Spores from 5-d-old cultures grown on PDA slant at 30°C were suspended in 5 mL of sterile 50 m*M* phosphate buffer (pH 6.8) containing 0.1% Tween-80. This conidial suspension (10^{10}–10^{12} conidia/mL) was used as inoculum for GOX fermentation. The 2% inoculum was used for a 500-mL Erlenmeyer flask containing 100 mL of basal medium: 1.0 g/L of $(NH_4)_2HPO_4$, 0.5 g/L of KH_2PO_4, 0.15 g/L of $MgSO_4 \cdot 7H_2O$, 1.0 g/L of peptone, and 40 g/L (sterilized separately) of $CaCO_3$. The previously diluted and filter-sterilized (0.45-µm Millipore membrane) crude hydrolyzed molasses (corresponding to ~285 g of total reducing carbohydrates [trc]/L) and HCF-TM (corresponding to ~285 g of trc/L) was added to a final concentration equivalent to 12% glucose at an initial pH of 5.5 and 30°C unless $CaCO_3$ was added in the medium (pH 6.5 ± 0.1). The flasks were incubated in an orbital incubator shaker (Sanyo, Gallenkemp, UK) at 150 rpm under submerged fermentation at 30°C for 168 h. Unless otherwise indicated, culture samples were taken periodically, centrifuged (6000*g*, 10 min) and the supernatants were used for enzyme analysis. The growth of the mutant and WT culture was analyzed by determination of mycelium dry weight.

Determination of GOX and Residual Sugar

Samples (5 mL) were periodically withdrawn and filtered to analyze GOX activity and total protein concentration. GOX activity was determined as in ref. *16*. The enzyme reaction was carried out in a final volume of 750 µL containing 20 m*M* sodium phosphate buffer (pH 6.0), 0.7 m*M* *o*-dianisidine, 100 m*M* D-glucose, 20 µg/mL of HRP, and 100 µL of diluted sample. Enzyme reactions were performed for 10 min at 25°C and stopped with an equal volume of 4 *M* HCl solution. The absorbance of the reaction sample was measured at 540 nm. The values were converted to GOX activity (U/mL) by comparison with a standard curve of 0–0.05 (U/mL) prepared with commercially available GOX (Sigma). The amount of enzyme required to oxidize 1 µmol of glucose/min at 25°C and pH 6.0 was defined as 1 U of GOX activity. Total protein concentration was measured by the Lowry method *(17)* using bovine serum albumin as a standard. The unfermented total residual sugar was determined according to Miller *(18)*, and the total reducing carbohydrates (t.r.c.) was estimated as described in ref. *19*.

Determination of Dry Cell Mass

The culture fluid was filtered through Whatman No. 1 paper. The filtered mycelia were washed with acidified (pH 2.5 with 4 *N* HCl) ddH_2O to convert the insoluble $CaCO_3$ to soluble $CaCl_2$. The separated mycelia were washed several times with deionized water until the pH of the washing was 7.0; mycelia were then dried at 75°C to constant weight after repeated weighing.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) of the mutated fungal mycelia was performed by fixing with 2% glutaraldehyde. After dehydration in ethanol series (30–100%), the samples were air-dried, coated with gold, and examined using a scanning electron microscope (Leo 435VP). The mycelia of WT *A. niger* ORS-4 were processed and observed in a similar manner.

Reproducibility of Results

All fermentations were carried out in triplicate and the experimental results represent the mean of three identical fermentations. One-way analysis of variance tests followed by least significant differences (Tukey's Honestly Significant Difference) were performed on GOX production to evaluate the potential significant differences between GOX production levels.

Results

Screening for Potent Microbial Strain

Soil from sugarcane industry waste-dumping sites was used to isolate the fungal strains. Among the strains isolated, seven distinct colonies that appeared on the yeast extract agar medium and showed a wider brownish red zone on *o*-anisidine-containing agar plates were selected (Table 1). The selected strains were further grown in HCF-TM-substituted medium in submerged culture conditions and analyzed for total GOX activity (Table 1). Maximum activity was shown by strain ORS-4 (1.05 ± 0.23 U/mL), which was identified as *A. niger* and therefore selected for further studies; the other six isolated strains showed less GOX activity in liquid culture medium (Table 1). Strain *A. niger* ORS-4 was one of the seven isolates, and although it had a comparatively lower growth rate, it exhibited higher levels of GOX activity (1.05 ± 0.23 U/mL) in the fermentation medium. To enhance extracellular GOX levels in the fermentation medium, the organism *A. niger* ORS-4 was further subjected to physical mutagenesis using UV irradiation.

UV Mutation and Screening of A. niger *Mutants*

Two-step UV irradiation was used to stimulate GOX activity of *A. niger* ORS-4 (Fig. 1). The optimal dose of UV irradiation was determined by treating the nascent fungal spores with varying doses of UV and analyzing

Table 1
GOX Activity of Microbial Strains From Sugarcane Industry Wastes in Liquid Culture Using HCF-Treated Sugarcane Molasses as Cheap Carbohydrate Source

Strain	GOD activity (U/mL)[a]	Cell dry weight (mg/mL)[a]	Fermentation time (h)[b]
ORS-1	0.340 ± 0.012	9.6 ± 2.0	120
ORS-2	0.070 ± 0.023	10.1 ± 2.8	120
ORS-3	0.048 ± 0.023	8.7 ± 1.9	96
ORS-4	1.050 ± 0.23	8.2 ± 1.6	144
ORS-5	0.038 ± 0.017	9.5 ± 2.4	144
ORS-6	0.054 ± 0.019	6.8 ± 0.9	72
ORS-7	0.024 ± 0.010	4.8 ± 0.5	120

[a]Values represent the means of three replicates ± SD.
[b]Fermentation time taken to reach maximum enzyme activity.

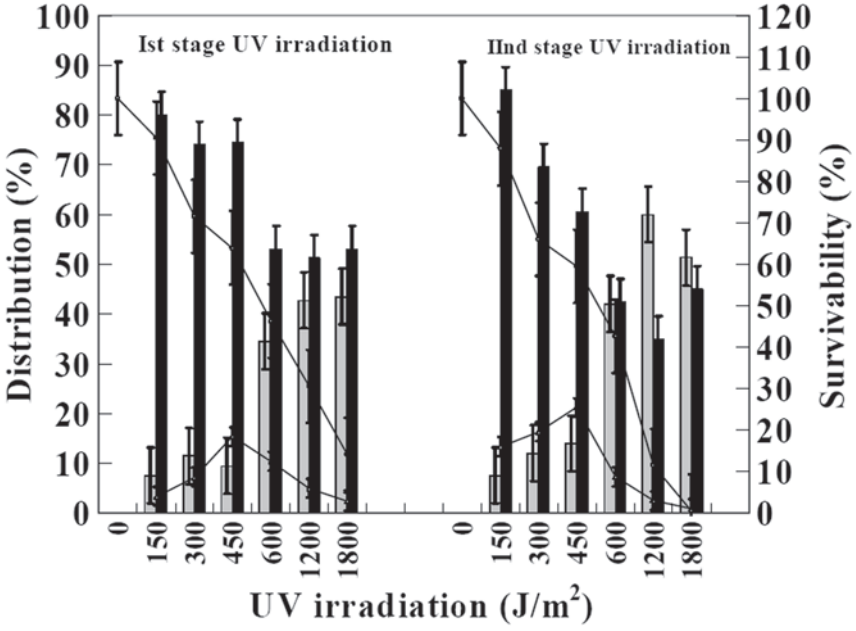

Fig. 1. Analysis of UV irradiation on conidial survival (%) and distribution (%) of WT *A. niger* ORS-4 (*left*) and of *A. niger* mutant ORS-4.110 (*right*) colonies on basis of diameter (mm) of brown halos produced by GOX diffusion around colonies. The 3-d-old WT *A. niger* ORS-4 spores were exposed under varying degrees of UV irradiation (left), and the selected mutant after first-stage UV exposure was reexposed by a second stage of varying degree of UV irradiation. The rates of survivability (○), positive mutants (△), negative mutants (solid bars), and corresponding mutants (shaded bars) were selected as described in Materials and Methods. Values are the mean ± SEM expressed from a total of three individual parallel experiments.

Table 2
GOX Activity of WT *A. niger* ORS-4 and of Most Active Mutants Obtained After Mutagenesis of First-Stage UV Mutated Strain *A. niger* ORS-4.110 in Submerged Fermentation Using HCF-Treated Sugarcane Molasses

Strain of *A. niger*	Size of diffusion halos for GOX activity (mm)[a]	GOX activity in liquid culture (U/mL)[b]
ORS-4	3.5	1.05 (100)
ORS-4.395	7.9	2.21 (210)
ORS-4.410	11.8	2.62 (249)
ORS-4.458	7.0	1.70 (161)
ORS-4.515	8.6	2.25 (214)
ORS-4.567	9.3	2.32 (220)
ORS-4.618	6.5	1.30 (123)
ORS-4.779	7.7	1.90 (180)
ORS-4.821	7.9	2.15 (204)
ORS-4.866	7.6	1.85 (176)
ORS-4.931	7.8	1.97 (187)
ORS-4.989	9.5	2.37 (225)

[a]GOX activity is expressed as the diameter (mm) of enzyme diffusion zones around the colonies on double-layered agar plate.

[b]Relative GOX activity (%) compared with the activity of the WT strain (taken as 100%) is given in parentheses.

the effects with respect to conidial survival and the frequency of positive and negative mutants. The mutant ORS-4.110 that resulted after the first stage of UV irradiation from parental strain ORS-4 was selected and reexposed to UV for second-stage mutagenesis. The percentage of cellular survivability was enumerated after each dose of UV irradiation (Fig. 1). In both cases, a UV dose of 450 J/m^2 appeared suitable and resulted in 16 and 25.5% positive mutants after the first and second stage of UV exposure, respectively. The cell survivability obtained was 61% in the first stage of UV treatment and 57% after the second stage (Fig. 1). Higher doses of UV irradiation retarded both the frequency of positive mutants and cell survivability. UV doses beyond 450 J/m^2 led to an increase in negative mutants; however, the number of corresponding mutants dropped. The level of GOX liberation from irradiated conidia was primarily detected by measuring the diameter of the brown halos of oxidized *o*-dianisidine around the colonies on double-layered agar plates (Table 2).

GOX Activity and Mutant Stability

Of about 1500 colonies tested, 45 colonies from the first-stage UV mutant *A. niger* ORS-4.110 showed enzyme diffusion halos greater than 5.7 mm in diameter (Table 2) after second-stage UV mutation. Based on the size of the oxidized *o*-dianisidine diffusion zone around the irradiated colonies from the second-stage mutation, 50 mutated colonies were selected to obtain the enzymatic levels in submerged culture cultivation process with TM-substituted fermentation medium (Fig. 2). Most of the mutants

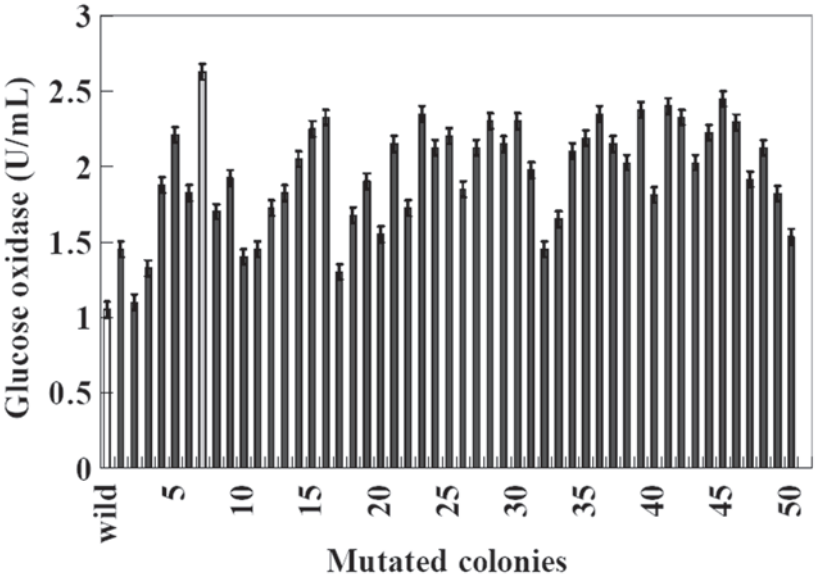

Fig. 2. Level of GOX activity from second-stage UV mutated *A. niger* colonies (solid bars) in HCF-treated sugarcane-substituted fermentation medium. These colonies were derived from first-stage UV mutant *A. niger* ORS-4.110 and compared with those of WT *A. niger* ORS-4 (open bar). The second-stage UV mutant *A. niger* ORS-4.410 (shaded bar) represented the highest level of GOX activity. Values are the mean ± SEM expressed from a total of three individual sets of experiments.

showed higher levels of GOX (1.10–2.62 U/mL) than the WT strain ORS-4 (1.050 ± 0.23 U/mL) (Fig. 2). Mutants ORS-4.410 and ORS-4.989 had the maximum activity levels (2.62 ± 0.51 and 2.37 ± 0.68 U/mL, respectively), which are 149 and 125% higher than that of the WT *A. niger* ORS-4 (Table 2). Among the other mutants selected, the levels of GOX activity for five mutants increased by 23–87%, and three mutants had 104–114% increases in activity level (Table 2). The strain *A. niger* ORS-4.410 was used for further experiments.

The stability of an organism in any fermentation process plays a vital role in the longer-term operation of a fermentation industry. In the present study, the stability of GOX production by mutant strain ORS-4.410 was monitored continuously. Mutant *A. niger* ORS-4.410 was subcultured monthly for 24 mo and each month represents a new generation of mold ORS-4.410. GOX fermentation reactions from each new generation were carried out continuously over 24 mo (Fig. 3) and showed a respectable consistency in GOX levels with minor statistically significant variations.

Morphologic Characterization of WT and Mutant A. niger

Figure 4 shows the characteristic features of the WT *A. niger* ORS-4 and mutant strain ORS-4.410. Distinct changes in color can be seen in the mycelia of the fungal strains. However, the spores produced by the mutants obtained after UV mutagenesis appeared to be black, just as in the parental

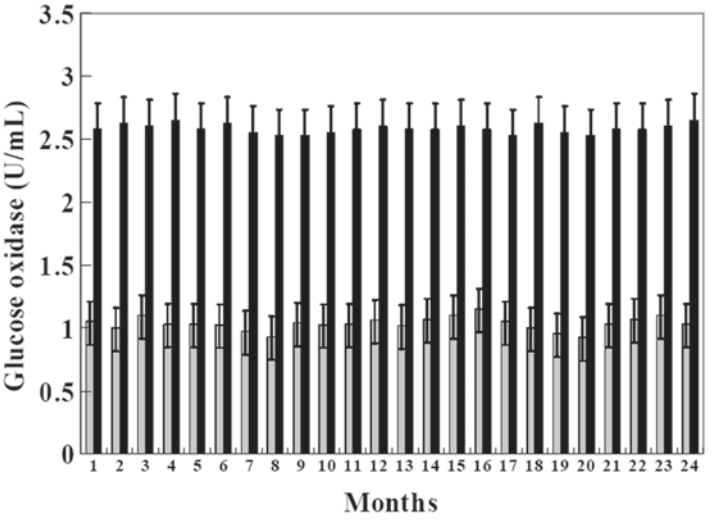

Fig. 3. Stability in GOX production by mutant *A. niger* ORS-4.410 and WT *A. niger* ORS-4. The second-stage UV mutant *A. niger* ORS-4.410 (solid bars) and WT *A. niger* ORS-4 (shaded bars) were transferred to new generations over a period of 24 mo for 24 generations, and the levels of GOX production stability were detected from every generation in HCF-treated sugarcane molasses-supplemented fermentation medium. Values are the mean ± SEM expressed from a total of three individual sets of experiments.

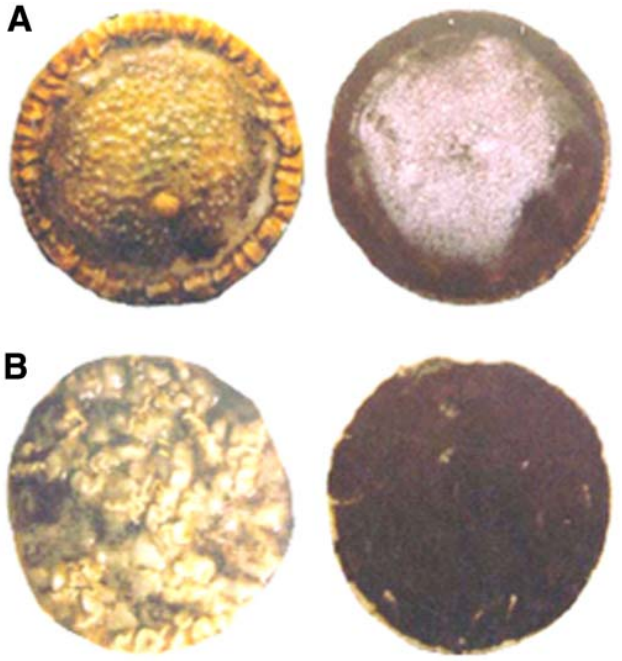

Fig. 4. Morphological variations between WT *A. niger* ORS-4 and mutant *A. niger* ORS-4.410 under static culture conditions. **(A)** (Left) Reverse side of mycelium, i.e., side in contact with fermentation medium supplemented with HCF-treated sugarcane molasses, indicating a yellow mycelium of *A. niger* ORS-4 and (*right*) upper side of same culture, indicating extent of sporulation; **(B)** (*left*) dirty white mycelium of mutant *A. niger* ORS-4.410 on reverse side under same culture conditions and (*right*) heavy sporulation on upper side of same culture.

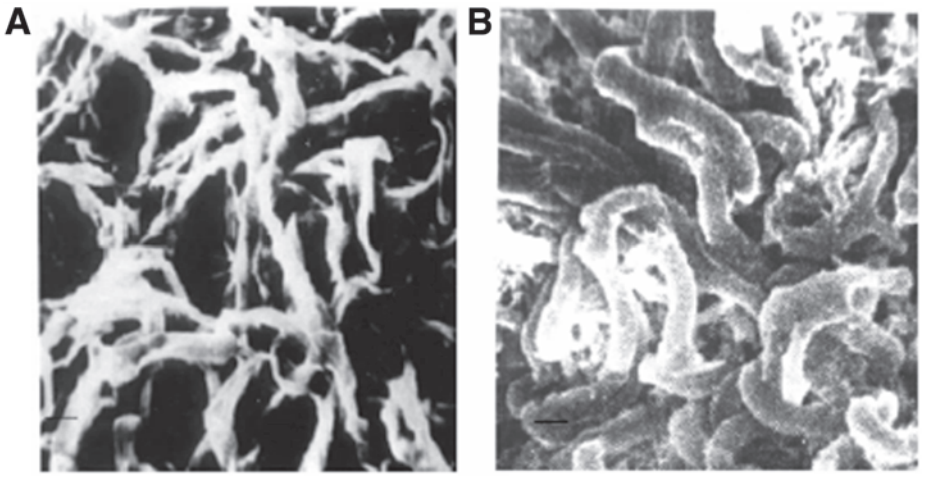

Fig. 5. Scanning electron micrograph of **(A)** WT *A. niger* ORS-4 and **(B)** mutant *A. niger* ORS-4.410 grown in liquid culture at 150 rpm and 30°C in defined basal medium. After the desired state of fermentation, mycelia from WT *A. niger* ORS-4 and mutant ORS-4.410 were separated and prepared for SEM as described in Materials and Methods. The morphologic variations in WT and mutant *A. niger* ORS-4 and ORS-4.410, respectively, were acquired at equal magnification: ×1500 (scale = 10 μm). (Adapted from ref. *15*.)

strain. The mycelia of WT strain *A. niger* ORS-4 were brownish yellow (Fig. 4A) and had a compact growth, whereas the mycelia of ORS-4.410, obtained after the second stage of UV irradiation, were dirty white (Fig. 4B).

Figure 5 shows the fine structural detail of the mycelia, as observed through a scanning electron microscope. The hyphae of the WT *A. niger* ORS-4 are compact and seen in the form of clumps (Fig. 5A). The mutant ORS-4.410 obtained after two steps of UV irradiation had thick, short, and globular hyphae (Fig. 5B). The corelationship between distinct morphological features and GOX production has not been worked out; this requires in-depth biochemical and molecular analysis for a notable induction of GOX activity in *A. niger*.

Fig. 6. *(opposite page)* Utilization of sugarcane molasses for GOX production. Batch fermentation was performed at pH 6.5 ± 0.1 and 30°C. Mutant *A. niger* ORS-4.410 was grown in fermentation medium supplemented with crude sugarcane molasses (CM) and HCF-treated clarified form of sugarcane molasses (TM). After the desired time period of fermentation reaction, 5 mL of medium was withdrawn and analyzed for **(A)** total GOX activity in TM (solid bars) and CM (open bars), total specific GOX activity in TM (○) and CM (△)–supplemented fermentation medium; **(B)** total cellular dry mass in TM (solid bars) and CM (open bars), total protein in TM (○) and CM (△)–supplemented fermentation medium; and **(C)** unfermented total residual sugars in TM (○) and CM (△). Values are the mean ± SEM expressed from a total of three individual experiments. Extremely significant differences were observed ($p = 0.000$) between total GOX activity at different fermentation time intervals using TM and CM followed by the least significant differences ($p < 0.05$).

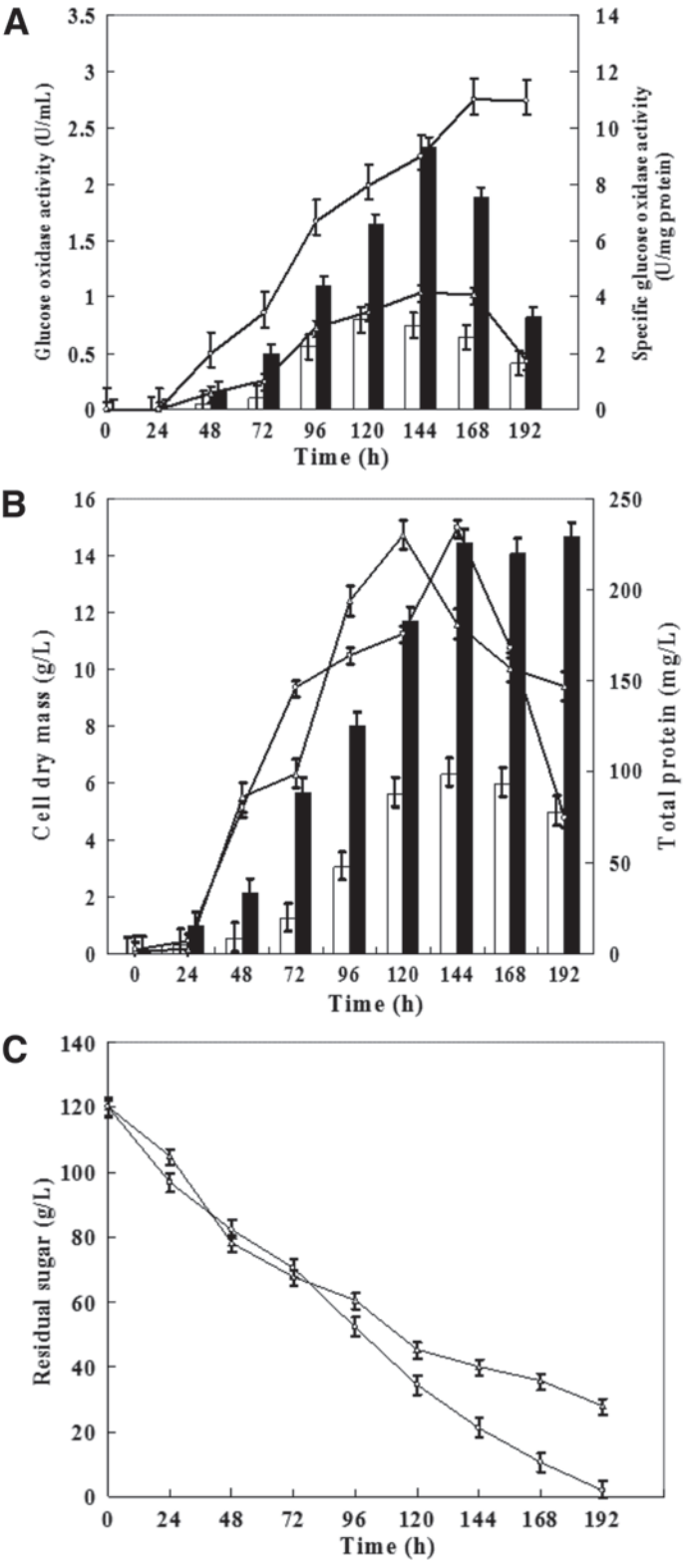
A
Glucose oxidase activity (U/mL)
Specific glucose oxidase activity (U/mg protein)
Time (h)
B
Cell dry mass (g/L)
Total protein (mg/L)
Time (h)
C
Residual sugar (g/L)
Time (h)

Fig. 6.

GOX Production From Sugarcane Molasses-Substituted Fermentation Medium

As an unconventional carbohydrate substrate for GOX production, we used sugarcane molasses, a cheaper carbohydrate source that is utilized for a variety of fermentation processes *(15,20,21)*. During the course of our studies using *A. niger* mutant ORS-4.410 for extracellular GOX production, CM resulted in lower levels of GOX (0.720 ± 0.158 U/mL); however, a 3.43-fold increase in GOX level was observed with TM (2.620 ± 0.51 U/mL) (Fig. 6A). Statistically, the significant differences were found in between GOX production using CM and TM (CM: $F = 37.94$; df = 7, 16; $p = 0.000$; TM: $F = 57.31$; df = 7, 16; $p = 0.000$), followed by the least significant difference ($p < 0.05$). During the fermentation reaction, a steady increase in specific GOX activity was observed in TM (11.01 U/mg of protein), compared with CM (4.07 U/mg of protein) (Fig. 6A). Interestingly, the total protein content in CM increased exponentially (194.21 mg/L) after 96 h and continued for up to 120 h of fermentation (230.58 mg/L); further incubation of mutant *A. niger* ORS-4.410 in CM sharply decreased the protein content (Fig. 6B). During the course of fermentation, gradual decreases in residual sugars were observed in CM and TM. However, mutant *A. niger* ORS-4.410 did not seem to utilize the total carbohydrate source beyond 120 h in CM-supplemented fermentation medium and left a significant amount of sugar (36.21 g/L) (Fig. 6C).

Discussion

GOX-Producing Microorganism

A stable microbial strain having characteristics of producing the enzyme of interest is a prerequisite for any successful fermentation industry. Because most of the work regarding isolation and improvement of microorganisms is done at industrial research and development centers, detailed information on the strains used is often not disclosed *(15)*. Nevertheless, the general strategy is to screen the naturally occurring wild strains for their ability to produce enzymes. In this process, it is not uncommon that investigators stumble on organisms with multiple capabilities. Fiedurek and Ilczuk *(22)* screened 1486 mold strains in search of extracellular GOX producers and found 119 active GOX producers, mainly belonging to the *Aspergillus* and *Penicillium* groups. Later, new strains of *A. niger (13,23)* with better GOX activity were identified. It has been generally observed that only selected strains of *A. niger* are best suited for extracellular GOX production *(1,13,22–27)*. Therefore, we attempted to select a microbial strain for extracellular GOX production from an area where sugarcane industry wastes were continually being decomposed. Among the various isolated strains, we selected seven strains with the widest brown halos of oxidized *o*-dianisidine around the colonies on a two-layered agar plate system. In a similar attempt, microbial strains actively producing

GOX have been isolated from different natural resources *(22)*. Among the seven strains isolated in our study, ORS-4 (identified as *A. niger*) was found to be the best, producing a zone that was two to three times larger than the zones obtained for any other fungus (Table 1). This strain was able to produce a good amount of extracellular GOX, but the rate of GOX production was slow. Unfortunately, the expectations for commercial exploitation of this strain did not turn out to be viable, owing to moderate sporulation and growth rate. Strain improvement was therefore attempted by physical mutagenesis, using varying doses of UV irradiation to enhance the characteristics of *A. niger* ORS-4 that could make the process economical and efficient using sugarcane molasses as a cheaper carbohydrate source in the fermentation medium.

Strain Improvement by Mutagenesis and Mutant Stability

The strain ORS-4 was subjected to varying degrees of UV irradiation in two steps, and the numbers of positive and negative mutants after each step of mutagenesis were scored on the basis of brown halo size. As expected, UV irradiation of *A. niger* produced both positive and negative mutants. The proportion of negative mutants after first-stage UV mutagenesis was lower compared with the second stage of UV exposure (Fig. 1). The WT parental strain used for the first UV exposure may perhaps have a greater stability to UV radiation than the mutant strain ORS-4.110 used for the second-stage UV exposure. The viabilities of the colonies obtained after the first and second stages of UV exposure were 61 and 57%, respectively (Fig. 1). These results are in agreement with those reported earlier for GOX-overproducing mutants of *Penicillium variabile* and *Penicillium canescens* mutants overproducing glycosidases *(9,28)*. In both of these studies, the higher frequencies of positive mutants were obtained with a viability range varying between 30 and 65%. Witteveen et al. *(1)* also identified GOX-overproducing mutants at conidial survival rates ranging from 33 to 78%. Among the various mutants obtained, *A. niger* ORS-4.410 was found to have the widest brown halo zone and maximum GOX-producing capacity in liquid culture (Table 2, Fig. 2).

Owing to the nonspecificity of UV mutagenesis, obtaining desired mutants after single, multiple, and varying degrees of UV exposure is a matter of chance, as is the stability of each particular mutant. Mutant *A. niger* ORS-4.410 was subcultured every month on PDA medium for a total of 24 mo in a new culture slant. Over the 24-mo period, each new generation represented a renewed growth cycle every month for mutant *A. niger* ORS-4.410 following subculturing. During the entire course of our study of 24 mo and/or 24 generations, mutant *A. niger* ORS-4.410 showed consistency in GOX production under submerged fermentation conditions (Fig. 3). Statistically, minor variations in GOX levels were found in successive generations of the mutant ORS-4.410, indicating that the mutant was just as stable as the WT strain.

Morphological Characterization

Mutagenesis not only resulted in strains with varied GOX production abilities, but also changed morphologic features of the molds. For instance, *A. niger* ORS-4 had a compact mycelium that was yellow with moderated sporulation, whereas the mycelium of ORS-4.410 was noticeably dirty white with thick sporulation (Fig. 4A,B). The yellow color in *A. niger* has been attributed to the pigment asperenone *(29)*. From the results, it is evident that UV exposure brings about discoloration of the mycelium. It may therefore be inferred that UV exposure perhaps interferes with the biosynthesis of asperenone. Whether the presence of asperenone is related to the production of GOX needs to be investigated further.

Evaluation of Sugarcane Molasses for GOX Fermentation

In the fermentation industry, the overall cost of a product is mainly determined by the cost of raw materials used during operation. To economize the process, sugarcane molasses has been considered as an unconventional carbohydrate source in various fermentation processes *(15,20,21)* and was evaluated here for extracellular GOX production using mutant *A. niger* ORS-4.410. Molasses represents a highly complex system that picks up a variety of heavy metal ions during the sugar crystallization and cleaning process *(15,30)*. It was not found suitable for fermentation reactions, because heavy metal ions, especially Fe^{3+}, Cu^{3+}, Zn^{2+}, and Mn^{2+}, are strongly inhibitory to fermentation reactions *(6,31)*. Therefore, these heavy metal ions must be removed, which can be achieved by treating the molasses with appropriate concentrations (3.8 m*M*) of HCF. HCF is known to precipitate most of the heavy metals that can easily be removed by filtration *(15,30)*. The clarified substrate, TM, is suitable for GOX fermentation and has led to a 3.43-fold increase in GOX production by *A. niger* mutant ORS-4.410 (Fig. 6). This influence stems from the fact that 3.43 times more GOX production is possible using HCF-treated molasses than using untreated sugarcane molasses. Although clarification of these carbohydrate sources leads to better utilization of substrates, a depletion of essential nutrients/ions also occurs. Therefore, many of these essential nutrients need to be added in appropriate concentration before fermentation with *A. niger* ORS-4.410.

These observations therefore substantiated that sugarcane molasses can be a potential substrate for GOX production. Depending on the degree of clarification for removal of heavy metal ions, varying amounts of unfermented sugars would remain unused in the fermentation medium (Fig. 6C). Attempts have been made to utilize xylane and corn steep liquor with *Aspergillus nidulans* and *A. niger*, respectively, for GOX production *(13,14)*. However, using CM and TM from the present study would pave the way to economize the GOX fermentation process. Our study thus reveals that *A. niger* ORS-4.410 can be an effective and promising mold for GOX production using the agroindustry byproduct of sugarcane molasses as a cheap carbohydrate source.

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