Gluconic acid production by *Aspergillus niger* mutant ORS-4.410 in submerged and solid state surface fermentation

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*Aspergillus niger* ORS-4.410, a mutant of *Aspergillus niger* ORS-4 was produced by repeated irradiation with UV rays. Treatments with chemical mutagens also resulted into mutant strains. The mutants differed from the parent strain morphologically and in gluconic acid production. The relationship between UV treatment dosage, conidial survival and frequency of mutation showed the maximum frequency of positive mutants (25%) was obtained along with a conidial survival of 59% after second stage of UV irradiation. Comparison of gluconic acid production of the parent and mutant ORS-4.410 strain showed a significant increase in gluconic acid production that was 87% higher than the wild type strain. ORS-4.410 strain when transferred every 15 days and monitored for gluconic acid levels for a total period of ten months appeared stable. Mutant ORS-4.410 at 12% substrate concentration resulted into significantly higher i.e. 85-87 and 94-97% yields of gluconic acid under submerged and solid state surface conditions respectively. Further increase in substrate concentration appeared inhibitory. Maximum yield of gluconic acid was obtained after 6 days under submerged condition and decreased on further cultivation. Solid state surface culture condition on the other hand resulted into higher yield after 12 days of cultivation and similar levels of yields continued thereafter.

D-gluconic acid, an oxidative product of D-glucose, is extensively used in food, feed and pharmaceutical industry\(^1\). There are several reports on fermentative production of gluconic acid and its salts by various bacterial and mould species. The commonly studied bacterial species are *Pseudomonas, Acetobacter*\(^2\) and *Glucunobacter*\(^3\) while in moulds *Penicillium*\(^4\) and *Aspergillus*\(^5\) have been considered suitable for commercial exploitation for the purpose. Attempts have been made for developing improved calcium gluconate producing strain *viz.* *Penicillium funiculosum* PS2, *A. niger* AB1801\(^7\) by physical and chemical mutagenesis. Further, a gluconic acid and glucose oxidase overproducing mutant *Penicillium variable* M 80.10 has been developed from *Penicillium variabile* P16 through UV mutagenesis\(^8\). Total worldwide production of calcium and sodium salts of gluconic acid is estimated to be approximately 50,000-60,000 ton annually and has thus emphasized various groups for extensive investigations towards identifying and developing potent microbial strain and bioprocesses for industrial production of gluconic acid.

Our aim has been to use the sugarcane industry wastes for selecting microbial strains that may significantly be used for gluconic acid production. We have, therefore, selected the site decomposed with the sugarcane industry waste and isolated the strain, *Aspergillus niger* ORS-4 that has been found to have a remarkable ability for gluconic acid production with a total yield of 75-80% (Ref. 9). Unfortunately the strain is slow growing with moderate sporulation and therefore has a disadvantage for commercial utilization of the strain. We have, therefore, attempted the two step physical and chemical mutagenesis to develop a suitable mutant of *A. niger* ORS-4 and has analyzed the parameters for culture conditions so as to use the strain for commercial production of the gluconic acid.

**Materials and Methods**

**Microorganism**—The wild type strain, *Aspergillus niger* ORS-4 was isolated from the site of decomposed sugarcane industry wastes\(^9\) and maintained on potato dextrose agar (PDA) slants by periodical transfers.

**Mutagenesis and selection**—Two step physical mutagenesis was carried out using the spore suspension of *A. niger* ORS-4 (10 mL, \(1\times10^9\) spore/mL) in petri dish by UV illumination (2.5 J/m²/s, distance 0.69 m) for different times ranging from 60 sec to 12 min. The conidial suspension (\(1\times10^9\) spore/mL) of *A. niger* ORS-4 was treated with N-methyl-n'-nitro-N-nitrosoguanidine (MNGN, 100 µg/mL) for 10-60
min at 30°C. The hydrated conidial suspension of *A. niger* ORS-4 were also treated with 0.2 mL of sodium nitrate (5 mg/mL) solution for 10-60 min at 30°C, which generated the mutagenic agent, nitrous acid, in the sodium acetate buffer (1.8 mL, pH 4.4)\(^\text{10}\). Following every treatment, conidial survival after appropriate dilutions was determined on PDA plates at 30°C and selection of the surviving mutant strains was performed on optimized mineral salt agar medium containing (g/L): NaNO\(_3\), 2.5; KH\(_2\)PO\(_4\), 1.0; MgSO\(_4\).7H\(_2\)O, 0.5; KCl, 0.5; FeSO\(_4\).7H\(_2\)O, 0.01; glycerol, 15.0; glucose, 20.0; agar, 15.0; with 0.04% alcoholic solution of bromocresol green used as an indicator for rapid screening of highly acidogenic strains on the basis of their acid unitage value\(^\text{6}\). The colonies thus selected were further streaked for purification on identical media.

**Growth in liquid medium and culture conditions**—Purified spores from 5 days old cultures grown on PDA slants at 30°C were suspended (10\(^{10}\)-10\(^{12}\) spores/mL) in 5 mL of sterile 0.05 M phosphate buffer (pH 6.8) containing 0.1% Tween-80 and 2% of inoculum was used for 250 mL of Erlenmeyer flask containing 50 mL of culture medium containing (g/L): (NH\(_4\)\(_2\))\(_2\)HPO\(_4\), 1.0; KH\(_2\)PO\(_4\), 0.5; MgSO\(_4\).7H\(_2\)O, 0.15; CaCO\(_3\) was added in the medium (pH 6.5 ± 0.1) with continuous shaking (150 rpm) for submerged fermentation process. The solid state surface culture condition was evaluated by using bagasse as the solid support. Before use, the finely powdered bagasse (obtained from sugar mill) was treated overnight with 2N HCl at room temperature, followed by thorough washing with double distilled water till the wash gets neutralized. A slurry of medium containing 15% of treated bagasse (70% moisture contents) was inoculated with 5 mL of *A. niger* spores (as stated above) and incubated for 12 days at 30°C, with occasional shaking to ensure a surface culture; waste culture cultivation gases were removed by connecting the culture flask to the water suction pump with the circulation of fresh air over the fungal mat on solid surface. After different time intervals the samples were collected by filtration for chemical analysis and growth was followed by mycelium dry weight determination\(^\text{11}\).

**Chemical analysis**—The unutilized total residual sugar concentration was assayed according to Miller\(^\text{12}\). Gluconic acid formed was determined by assaying the dissolved calcium amount in culture medium\(^\text{13}\). The broth containing gluconic acid was subjected to acid hydrolysis and resulting gluconolactone was measured by the modified hydroxamate method\(^\text{14}\).

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

All the experiments were performed in triplicate and the experimental results represent the mean of three identical cultivations.

**Results and Discussion**

The fungal strain used for the present investigation was isolated from the sites decomposed with the sugarcane industry wastes and was characterized as *Aspergillus niger* ORS-4. This strain was one of the seven isolates and although comparatively had a lower growth rate but produced apparently higher level of gluconic acid. Strain ORS-4 was further subjected to physical and chemical mutagenesis for increasing the yield and production of gluconic acid.

**Mutagenesis and screening of *A. niger* ORS-4**—Two step UV irradiation and chemical mutagenesis were used for improving the gluconic acid levels produced by *A. niger* ORS-4. The optimal conditions for mutagenesis were derived by variations in UV and chemical mutagen exposures and by evaluating the conidial survival and the frequency of positive and negative mutants. The distribution of treated colonies according to the acid unitage value on mineral salt agar acid indicator media and the percentage viability have been shown in Fig. 1a-d. Among the range of dosage used for first and second stage UV irradiation, the second stage of UV irradiation (450 J/m\(^2\)) appeared to ensure the maximum overproducing mutants (25% of the tested colonies) with 59% of cell survival (Fig. 1b). UV irradiation, as expected, of *A. niger* ORS-4 cells yielded both positive and negative mutants. The proportion of negative mutants after first stage UV mutagenesis was low compared to the second stage of UV exposure and this may probably be due to the stability of the parental strain used for the initial UV exposure than the mutant strain ORS-4.410 used for the second stage UV exposure. Mutagenic treatments as observed earlier had resulted into glucose oxidase overproducing mutants of *P. variabilis*\(^\text{6}\) and *A. niger*\(^\text{15}\). The results observed showed that *A. niger* ORS-4.410, obtained after second stage of UV
Fig. 1—Analysis of mutagenesis on conidial survival (%), distribution (%) of Aspergillus niger ORS-4 colonies on the basis of AU value
Treatment given is (a)-1st stage UV irradiation; (b)-2nd stage UV irradiation; (c)-N-methyl-N'-nitro-N-nitrosoguanidine and (d)-Nitrous acid (—, survivability; □, negative mutants; ●, corresponding mutants; ■, positive mutants).

Table 1—Growth and gluconic acid production after physical and chemical mutagenesis of Aspergillus niger ORS-4 under submerged condition
[Values are mean ± SD of 3 replications]

<table>
<thead>
<tr>
<th>Mutagenic agent</th>
<th>Strains of A. niger</th>
<th>AU values</th>
<th>Gluconic acid (g/L)</th>
<th>Glucose consumption (g/L)</th>
<th>Yield (%)</th>
<th>Biomass (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>ORS-4</td>
<td>4.1±0.8</td>
<td>49.00(100)</td>
<td>64.6±7.6</td>
<td>75.6</td>
<td>9.5±0.8</td>
</tr>
<tr>
<td>1st Stage of UV</td>
<td>ORS-4.110</td>
<td>6.0±2.2</td>
<td>68.50(139)</td>
<td>110.2±8.8</td>
<td>62.1</td>
<td>14.3±15</td>
</tr>
<tr>
<td>2nd Stage of UV</td>
<td>ORS-4.410</td>
<td>8.3±1.8</td>
<td>91.79(187)</td>
<td>105.2±9.1</td>
<td>87.2</td>
<td>11.6±1.3</td>
</tr>
<tr>
<td>MNNG</td>
<td>ORSM-4.248</td>
<td>6.1±1.5</td>
<td>69.50(141)</td>
<td>105.6±9.5</td>
<td>65.7</td>
<td>14.8±2.8</td>
</tr>
<tr>
<td>Nitrous acid</td>
<td>ORSN-4.340</td>
<td>5.2±1.2</td>
<td>59.20(120)</td>
<td>97.3±7.2</td>
<td>60.8</td>
<td>15.8±2.3</td>
</tr>
</tbody>
</table>

a AU values expressed as diameter of yellow zone/diameter of mycelial zone.

b Values given in parantheses are the %age gluconic acid production as compared to wild type.
treatment (450 J/m²) had gluconic acid production, 87% higher than the wild type ORS-4 (Table 1). The gluconic acid overproducing mutant having such higher levels of acid production have not been described earlier⁸,¹⁶. The treatment with chemical mutagens i.e. MNNG and nitrous acid led to the mutants with marginally increased ability (20-40%) for gluconic acid production. The strain ORS-4.410, therefore, was employed for further investigation in the subsequent experiments. Product stability was monitored by subculturing the high acid producing mutant strain ORS-4.410 after every 15 days for a period of 10 months and simultaneously estimating the gluconic acid levels at respective intervals. Barring a minor variation, a stable gluconic acid production was observed after the strain ORS-4.410 was subcultured subsequently for 20 generations (Fig. 2).

Morphological characterization—Notable changes in the morphological features of the fungal mycelia following physical and chemical mutagenesis were observed as evident in the scanning electron microscopy of the wild type and mutagenized mycelia. The mutant ORS-4.410 after two stage UV mutagenesis had thick, short and globular mycelia (Fig. 3b) as compared to the mycelia of the wild type strain ORS-4 that was highly branched, smooth and elongated (Fig. 3a). So far the co-relationship between distinct morphological features and gluconic acid production has not been analyzed and probably the altered biochemical architecture and the activated glucose oxidase may lead to increased production of gluconic acid.

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Fig. 3—Scanning electron micrograph (SEM) of 72 hr old mycelia of (a) wild type Aspergillus niger ORS-4 and (b) mutant Aspergillus niger ORS-4.410 [Magnification x 1500 (Bar 10 μm)].
Analysis of factors affecting gluconic acid production by mutant *A. niger* ORS-4.410—Glucose in varying concentrations was used as substrate under submerged and solid state surface culture conditions for gluconic acid production and showed that 10-16% of glucose as substrate resulted into significantly higher production of gluconic acid by mutant strain *A. niger* ORS-4.410 (88-90 g/L) than parent strain (45-50 g/L) in submerged culture (Fig. 4a), whereas a further higher yield in gluconic acid production (98%) at 12% substrate concentration was observed in solid state surface culture condition (Fig. 4b) and was 78% higher than the wild type *A. niger* ORS-4 strain. The substrate below and above this concentration inhibited the acid production with a marginal increase in cell mass. It is assumed that sugars by osmosis enter in the fungal cell wall and the gluconic acid thus diffuses out. The low osmotic rate that may be generated at higher concentration of sugar may subsequently lead to a lower rate of gluconic acid diffusion due to the concentration gradient across the mycelial mat.

Comparatively lower growth for wild type *A. niger* ORS-4 was observed than mutant strain, *A. niger* ORS-4.410. The active growth of fungal mycelia of mutant strain ORS-4.410 began after 24 and 48 hr of incubation in submerged and surface culture condition respectively and was accompanied by the increasing production of gluconic acid.

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**Fig. 4**—Gluconic acid production by the mutant *Aspergillus niger* ORS-4.410 and the wild type *Aspergillus niger* ORS-4 at different glucose concentrations in (a) submerged and (b) solid state surface culture condition. (ORS-4.410: ▲, gluconic acid; ■, residual sugar; ●, biomass. ORS-4: △, gluconic acid; ◊, residual sugar; ○, biomass.)

**Fig. 5**—Production of gluconic acid at various intervals under (a) submerged and (b) solid state surface cultivation by mutant *Aspergillus niger* ORS-4.410 and wild type *Aspergillus niger* ORS-4. (ORS-4.410: ▲, gluconic acid; ■, residual sugar; ●, biomass. ORS-4: △, gluconic acid; ◊, residual sugar; ○, biomass.)
levels of gluconate production, the gluconate yield increased exponentially during active growth phase and a maximum acid yield upto 87% after six days and 95% after 12 days was obtained in submerged (Fig. 5a) and solid state surface culture condition (Fig. 5b) respectively. Beyond this period the gluconic acid concentration was found to drop in submerged condition whereas the continuous yield of gluconate production was obtained upto 25 days under surface cultivation process (data not shown) eventhough a very moderate increase in mycelial growth was observed beyond 10 days of incubation.

We have compared the yields of gluconic acid in solid state surface and in the submerged culture condition and former proved to be a better system for achieving higher yields for gluconate production. The addition of bagasse for solid state cultivation had considerably increased the surface area and hence aeration and thus inducing the oxidation process17, 18 for increased production of gluconic acid. Surface cultivation required longer periods for maximum yield but alternatively a system well equipped with agitation, aeration, foam and pH control, etc. may be suitably designed to achieve better yields in shorter time periods. The product generated remain stable in solid state process and higher with constant yield of production can be obtained for longer periods whereas the product was more susceptible for decomposition in submerged condition. Solid state surface culture condition, was comparatively simpler in operation involving much less power consumption and this simplicity in the operation made solid state culture process an attractive and economically viable proposition for gluconic acid production.

The above observation, therefore, indicated that a gluconic acid overproducing mutant A. niger ORS-4.410 was obtained and was stable after periodical transfer for several generations. The strain ORS-4.410, therefore, can be suitably utilized for large scale production of gluconic acid and in addition to the conventional process of production under submerged conditions, the solid state surface process can be economically employed for this purpose. Efforts are underway for scaling up the process in order to economize and for industrial exploitation of the developed strain.

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References